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REVIEW

Sulfate transport and metabolism: strategies to improve the seed protein quality

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Abstract

Sulfur-containing amino acids (SAA), namely methionine, and cysteine are crucial essential amino acids (EAA) considering the dietary requirements of humans and animals. However, a few crop plants, especially legumes, are characterized with suboptimal levels of these EAA thereby limiting their nutritive value. Hence, improved comprehension of the mechanistic perspective of sulfur transport and assimilation into storage reserve, seed storage protein (SSP), is imperative. Efforts to augment the level of SAA in seed storage protein form an integral component of strategies to balance nutritive quality and quantity. In this review, we highlight the emerging trends in the sulfur biofortification approaches namely transgenics, genetic and molecular breeding, and proteomic rebalancing with sulfur nutrition. The transgenic 'push and pull strategy' could enhance sulfur capture and storage by expressing genes that function as efficient transporters, sulfate assimilatory enzymes, sulfur-rich foreign protein sinks, or by suppressing catabolic enzymes. Modern molecular breeding approaches that adopt high throughput screening strategies and machine learning algorithms are invaluable in identifying candidate genes and alleles associated with SAA content and developing improved crop varieties. Sulfur is an essential plant nutrient and its optimal uptake is crucial for seed sulfur metabolism, thereby affecting seed quality and yields through proteomic rebalance between sulfur-rich and sulfur-poor seed storage proteins.

Keywords Sulfur-containing amino acids (SAA) · Sulfur transport and assimilation · Seed storage protein (SSP) · Biofortification · Transgenics · Genetic and molecular breeding · Sulfur nutrition

Introduction

To meet the calorie intake requirements of the growing population, modern agriculture techniques have revolutionized selective crop breeding and mechanized farming to improve crop yield, hardiness, and biomass production [1]. This focus on quantitative aspects of crop production has inadvertently affected the nutritional quality repertoire of the crops. Nevertheless, biofortification offers a sustainable solution for balancing the nutritive quality and quantity of the crops [2]. Contemporarily, there is a trend for preference for plant-based food, especially for proteins. Grain legumes are regarded as 'Poor man's meat', the key drivers for securing global food security, as the cheapest source of dietary protein and cradle of essential amino acids (EAA) [3]. Nevertheless, all-grain legumes are deficient in sulfur-containing amino acids (SAA) such as methionine and cysteine [1].

Sulfur is remarkably an interesting element because it transitions into a wide range of oxidation states, allowing sulfur-containing compounds to be involved in diverse biochemical reactions [4]. The deficiency of sulfur hinders the growth, development, disease resistance, and performance of plants and severely impedes the nutritional quality of crops [5]. Increasing sulfate assimilation and utilization efficiency is a viable approach for augmenting the concentration of SAA in legume seeds. Therefore, the biofortification of legume plant-based dietary proteins with limiting amino

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acids improves protein quality, thereby ensuring a balanced amino acid supply in the diet.

Cysteine and methionine are sulfur-containing amino acids that play an important nutritional role in both humans and animals. Although animals, except mammals, can biosynthesize cysteine from methionine, it is regarded as a conditionally essential amino acid. In contrast, methionine, being an EAA cannot be synthesized de novo via the transsulfuration pathway [6], is the first limiting amino acid that plays multifaceted cellular functions including the initiation of protein biosynthesis and acts as a methyl donor, in the form of S-adenosylmethionine (SAM), to phospholipids and polyamine [7]. The nutritional requirement of methionine in humans mainly depends on the physiological state (infancy, pregnancy, and lactation) and coordinative adjustments between methyl donors, methyl acceptors, and cysteine sparing effect. The phenomenon of dietary fulfillment of total sulfur amino acid by cysteine is called the cysteine-sparing effect. Though this notion is still debated, several publications have reported this effect ranging from 30 to 65% in both animals and humans. Certainly, a minimum obligatory requirement of methionine cannot be substituted by cysteine, which can be fulfilled exclusively by dietary intake of methionine-rich foods [8].

Cysteine is a crucial structural and functional component, present in all proteins and enzymes to an extent at least of 2% [9]. Cysteine and its derivatives such as Coenzyme A are major players in metabolic reactions such as essential fatty acid biosynthesis, cellular oxidative pathways (Tricarboxylic acid cycle, amino acid oxidation, and fatty acid β-oxidation), iron-sulfur biosynthesis, protein modification, and constituent of keratin and skeletal muscles [10]. It also serves as the repository of reduced sulfur in various cellular components such as homoglutathione, glutathione, iron-sulfur clusters, vitamin cofactors like biotin and thiamin, and multiple secondary metabolites [7].

A better comprehension of the metabolic pathways pertaining to sulfate transportation, assimilation, and their influence on SAA content within the seed protein reserve of legumes is of paramount significance. This review presents an overview of sulfur metabolism and discusses various approaches namely agronomical, molecular breeding, and genetic modifications to improve the content of SAAs in seed storage.

Sulfur metabolism

Sulfur is an essential macronutrient that exists in both organic and inorganic forms in the soil. Plants and microorganisms are primary assimilators, taking up inorganic sulfur in the form of sulfate (SO_4^{2-}) or sulfur dioxide and hydrogen sulfide gases either from soil or the atmosphere, respectively [11]. All other trophic organisms mainly depend on plant-derived, reduced, sulfur compounds. In recent decades, sulfur deficiency in agricultural lands has emerged as a serious concern due to poor cultural practices such as enhanced application of sulfur-free fertilizers and the dwindling use of traditional organic manures. The major available form of sulfur, inorganic sulfate, accounts for only 5% of total soil S in agricultural land, the remaining 95% is present in organically bound form. Organic sulfur such as sulfate ester or carbon-bonded sulfur potentially supports plant growth under sulfur-deficient conditions, indirectly by converting into inorganic sulfur through microbial mineralization [12]. Sulfate is the most oxidized and inert form of sulfur (+VI redox state), and its reduction, into sulfide, demands energy. Reduced sulfide is primarily incorporated into cysteine and different organic metabolites. Thus, sulfur in plants plays a multifaceted role by acting as a vital constituent of iron-sulfur proteins, vitamins (coenzyme-A, lipoate, thiamine), S-adenosylmethionine (methyl donor), glutathione (redox control), glucosinolates, and allins (defense role against herbivore), Choline-*O*-sulfate (osmoprotectant) [13]. Also, sulfur nutrition has a significant impact on nutritional quality, including oil content, and protein quality, of food crops, and is known to improve plant defense against biotic and abiotic stresses [14, 15].

Sulfate transporters

Sulfate is the major source of sulfur taken in soil and is transported into the plants by sulfate transporters present in roots, leaves, shoots, and seeds with a differing affinity for sulfate. These sulfate transporters are encoded by *SULTR* genes. Further, sulfate is assimilated into various essential organic thiols and sulfated organic compounds [4] as discussed later in this review. The complementation of a yeast mutant sulfate transporter by a cDNA library derived from a tropical legume, *Stylosanthes hamate*, led to the discovery of the first plant sulfate transporter [16]. The isolation and characterization of three different cDNA clones had proven the existence of multiple sulfate transporters in plants and function as high-affinity and low-affinity sulfate transporters depending upon different sulfate concentrations [16]. Further, the advancements in molecular biology and the availability of *Arabidopsis* genomic data (*Arabidopsis* Genome Initiative 2000) have assisted in the identification and isolation of the sulfate transporters gene family in *Arabidopsis* [17]. The sulfate transporters are encoded by multigene families, 14 genes in *Arabidopsis*, 13 genes in rice, and 10 in wheat, 9 in sorghum, and are classified into 4 distinct functional groups [18]. Further, sequence homology analysis confirmed that all the sulfate transporters in vascular plants are similar [19] posing many questions that the functional redundancy of a multigene family for these transporters is a consequence of gene duplication or are they encoded by individual genes with specific functions and whether this redundancy could offset the malfunction of the individual transporter? [20]. Gene duplications can now be identified by scrutinizing synteny relationships within the intragenomic or between cross-genome through PGDD (Plant Genome Duplication Database, http://chibba.agtec.uga.edu/duplication/). Even though, TDNA insertions could mutate the genes encoding SULTR1;1 and SULTR1;2 *Arabidopsis* still uptakes sulfate at a minimal rate through compensatory function by another member of the family [21] suggesting the functional redundancy. Nonetheless, most of the individual genes are differentiated by acquiring tissue-specific activity and variable responses to stress conditions. Thus, the knowledge of individual isoforms of the pathway and their function is invaluable for genetic manipulations-based precise regulation of sulfur metabolism in crops [19].

Structurally, sulfate transporter proteins have two domains, a 12 membrane-spanning helical domain (similar to membrane-bound solute transporters) and a STAS domain (Sulfate transporters and antisigma factor antagonist) [17]. The truncation of the C terminal region of SULTR1;2 STAS domain through deletion mutation compromised the ability of the transporter. Thus, STAS is a major regulatory domain responsible for protein-protein interaction, stabilization, and membrane localization of transporter proteins during sulfate transport [22].

Primary entry of sulfate against a negative gradient of membrane potential within roots occurs by the high-affinity group I transporters such as SULTR1;1 and SULTR1;2. Because of their affinity to sulfate ions (low Km 1.5 to 10 µM), they are predominately localized in the root hairs, epidermal and cortical cells of roots [23, 24]. The high-affinity group I transporters are primarily activated and expressed to take up sulfate under sulfate-deprived conditions. The sulfate and other anions in root epidermal cells move horizontally toward the central cylinder through symplastic plasmodesmal connections without traversing cell membranes [17]. Further, the translocation of sulfate from the central cylinder of the root to the shoot is mediated by low-affinity group II transporters (Km of 99.2 µM and 1.2 mM) [16, 25]. In corroboration with functional redundancy, PGDD database has identified that SULTR1;1 and SULTR2;2 are recently duplicated genes adjacent to SULTR1;2 and SULTR2;1 [19]. The duplicated genes do not equally contribute from the functional perspective and are differently regulated. SULTR1;2 is constitutively expressed while Sultr1;1 is highly responsive under sulfur-deprived conditions [4, 26]. Besides, tonoplast localized group IV transporters such as SULTR4;1 SULTR4;2 facilitate sulfate efflux from the vacuole and maintain symplastic flux level near SULTR2;1, before entering into the xylem [4]. Currently, limited information is available regarding the group III transporter. SULTR3;5 has been identified in *Arabidopsis* which is self-incapable to uptake the sulfate ion from apoplast. Hence it predominately co-localizes with SULTR2;1 and gradually increases the competence of the latter to uptake sulfate. Interestingly another group III homolog, SST1 identified in root nodules of *Lotus japonicas* and which remain localized in the symbiosome membrane. This nodule-specific sulfate transporter is indispensable for maintaining symbiosis between plants and rhizobia as in its absence, plants switch to non-symbiotic N- and S- assimilation pathways. Because sulfate is an essential nutrient of protein and metallocluster biosynthesis to constitute the metalloenzyme and nitrogenase, sulfate needs to be transported from host cell cytosol to intracellular rhizobia mediated by SST1 in a symbiotic process [26] (Fig. 1).

Sulfate reduction and metabolism

After the cellular entry, sulfate can either be stored temporarily in a vacuolar compartment or directly enters into the assimilation pathway in roots and leaves. In all the postulated sulfur assimilation pathways, the reduction of highly stable SO_4^{2-} to cysteine requires 14 ATPs accompanied by 10 electron transfers as it has a low reduction potential with an S oxidation state of + 6. Hence during various steps of the assimilatory pathway, several electron donors such as glutathione, thioredoxin, ferredoxin, NADPH, and OAS act concertedly at various steps [27]. Unlike the roots, leaves, being the site of photosynthesis and photorespiration, provide reducing equivalents (thioredoxin, ferredoxin) and OAS precursor (serine) [28] and act as a major active site of sulfur assimilation. The incorporation of sulfate into the bioorganic molecules in plants occurs either via the primary or secondary assimilatory pathway. Although both pathways diverge at a common intermediate, adenosine 5_-phosphosulfate (APS) as shown in Fig. 2, they end up with divergent products. In the former primary assimilatory pathway leading to cysteine, APS is reduced to sulfite (SO_3^{2-}) by APS reductase, which is further reduced into sulfide (S^{2-}) by sulfite reductase. In the next step, sulfide (S^{2-}) incorporation into the amino acid skeleton of OAS (O-acetyl serine) takes place to biosynthesize cysteine, the first product of the primary assimilation pathway. Further, cysteine serves as the precursor for the biosynthesis of methionine and all other metabolites containing reduced sulfur like glutathione, and phytochelatins. In the latter pathway, PAPS; a phosphorylated product of APS formed by APS kinase, serves as a sulfate donor for the sulfation reactions (Fig. 2). And thus, it is involved in the biosynthesis of an array of metabolites like

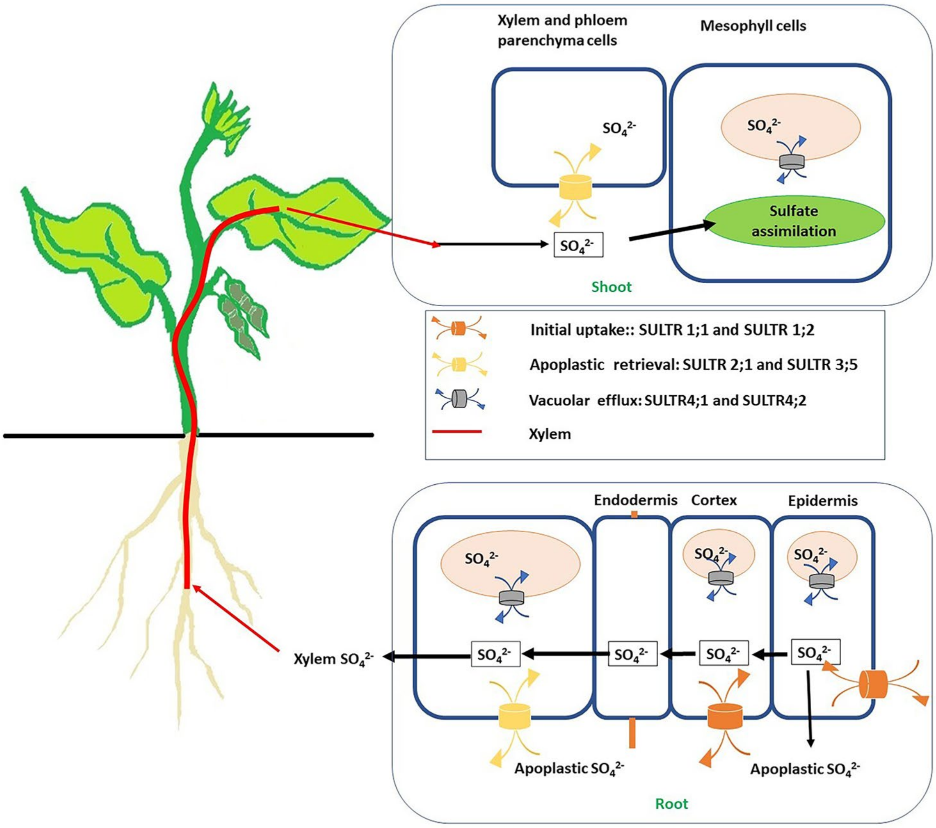

Fig. 1 Schematic representation of sulfate uptake and distribution within the plant system: Sulfate uptake in plant roots is primarily mediated by high-affinity group I transporters, SULTR1;1 and SULTR1;2. while translocation from roots and apoplastic space to root stele is facilitated by low-affinity group II transporters SULTR2;1 and supported by SULTR3;5. Sulfate is further transported into shoot through xylem tissue. While, tonoplast localized group IV transporters such as SULTR4;1 and SULTR4;2 facilitate sulfate efflux from the vacuole

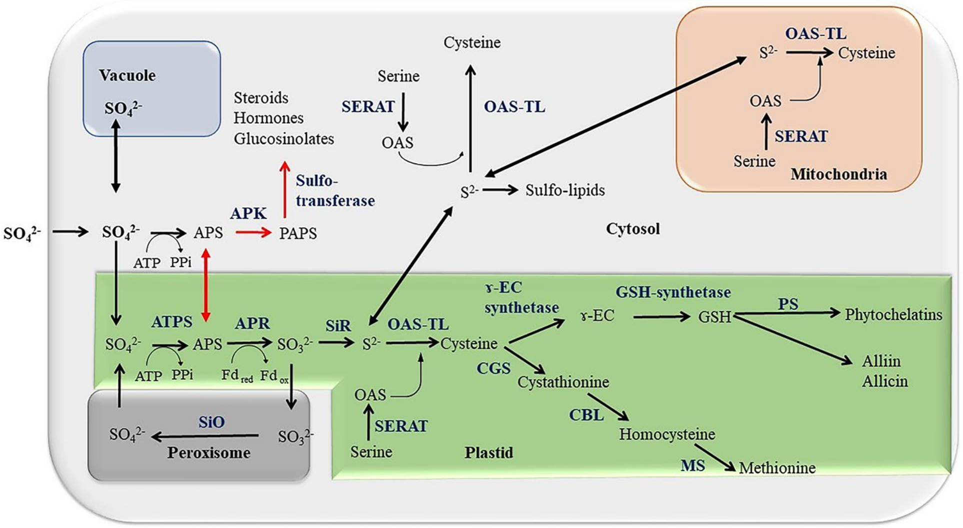

Fig. 2 Sulfur metabolism pathways in the plant: Sulfate uptake and distribution in plant facilitated by SULTRs, can temporarily in the vacuole. The primary sulfur assimilation pathway involves sequential biochemical reactions including chemical activation, reduction, and incorporation of sulfate into cysteine catalyzed by respective enzymes and the whole pathway is majorly located in plastids of roots and leaves. However, limited cysteine synthesis also occurs in mitochondria and cytosol by the virtue of isoforms for the respective enzymes. Cysteine in plastids further serves as the precursor for the biosynthesis of methionine, glutathione, and other metabolites. The secondary sulfur metabolism (indicated in red arrows) emerges after the activation of sulfate into PAPS which acts as a precursor to an array of metabolites in the cytosol. While sulfite oxidase localized in peroxisome serves a detoxifier role by recycling sulfate from sulfite. Abbreviations of metabolites; APS, adenosine 5’phosphosulfate; PAPS, 3_-phosphoadenosine 5_-phosphosulfate; OAS- *O*-acetyl serine; Abbreviations of enzymes: *ATPS*, ATP sulfurylase; *APR*, APS reductase; *APK*, APS kinase; *SiR*, sulfite reductase; *SERAT*, serine acetyltransferase; *OAS-TL*, OAS (thiol)lyase; *CBL*, cystathionine β-lyase; *CGS*, cystathionine γ-synthase; *MS*, methionine synthase; *SiO*, sulfite oxidase

brassinosteroids, glucosinolates, phytosulfokines, sulfojasmonate, and sulfoflavonoids [4].

Sulfate has low reduction potential, hence ATP-dependent activation of sulfate occurs by ATP sulfurylase (ATPS) and converts it into a chemically active form of APS. However, the formation of this high-energy phosphate-sulfate anhydride bond is thermodynamically extremely unfavorable and the equilibrium of the reaction strongly favors the reversal of the reaction. In plants, additive-free energy change that results from the coupling of inorganic pyro-phosphatase and ATP sulfurylase facilitates the forward reaction to generate APS [29]. Functional analysis in divergent sulfate assimilators, with a notable exception of *Selaginella moellendorffii*, indicated that all organisms have at least two isoforms of ATPS. In *Arabidopsis* and *Populus trichocarpa*, four isoforms of ATPS exist and function as dimeric enzymes [19]. ATPS isoforms are present in multicellular compartments; cytosol and plastid are relevant from genetic redundancy and physiological perspectives. The total ATPS activity of chloroplast in spinach was found to be 80% [30], suggesting that APS reduction majorly occurs in plastid. Cytosolic isoforms of ATPS may support secondary sulfate metabolism in coordination with APS kinase in PAPS biosynthesis [31]. In contrast to the genetic redundancy of sulfate transporters that resides adjacently in the genome, *ATPS* genes of *Arabidopsis* are spread on different chromosomes. However, the PGDD analysis revealed that the genes of *Arabidopsis*; ATPS1 and ATPS4 are located in duplicated segments of the genome [19].

Next, a glutathione-dependent reduction of APS into sulfite (SO_3^{2-}) and AMP is catalyzed by APS reductase (APR). In plants, APR is a rate-limiting enzyme of the primary sulfate assimilatory pathway and exhibits a substantially complex regulation. In *Arabidopsis*, APR transcription is regulated by salicylate, jasmonate, ethylene, and nitric oxide under salt stress. In plants, akin to ATPS, APR is also encoded by multigene families except for sorghum. Plant APR is a multidomain protein harbouring N-terminal reductase and C-terminal thioredoxin/glutaredoxin-like domains. The binding of the [Fe_4S_4] cluster as a cofactor to the reductase domain is indispensable for the APR activity. Interestingly, a recently discovered bacterial APR-like isoform, APR-B in *Sellaginella* and liverwort *Marchantia polymorpha* does not require [Fe_4S_4] cluster for its catalytic activity. Further, it lacks the thioredoxin/glutaredoxin domain and depends on free thioredoxin/glutaredoxin pools [19]. All these reports and phylogenetic analyses infer that FeS binding APR is an ancestral isoform that might have led to the evolution of APR-B in these basal plants under low iron availability conditions.

Sulfite reductase (SiR) is a monomeric enzyme that consists of a single FeS cluster and siroheme as prosthetic groups and utilizes ferredoxin as an electron donor. The transient ferredoxin-SiR complex catalyzes the selective six-electron transfer onto sulfite and thus reduces it into sulfide. In contrast to all other enzymes involved in sulfate assimilation in photosynthetic organisms, SiR is encoded by a single gene. In plant genomes, nitrate reductase (NiR) is known to be a paralogue of SiR, as both enzymes catalyze six electron reductions of their respective substrates, sulfate, and nitrate at different Km values. Thus, SiR and NiR might have evolved as a consequence of gene duplication events during the process of endosymbiosis. Alternatively, a peroxisomal localized enzyme, sulfite oxidase (SiO) oxidatively recycles sulfate from sulfite by transferring electrons onto molecular oxygen to form H_2O_2 with the aid of molybdenum-containing cofactor. However, its biological significance in sulfate flux in the cell remains to be elucidated. Its wide distribution among all higher plants suggests that it could function as a detoxifier of critical concentrations of either sulfur dioxide or superoxide anions during photosynthesis. Later sulfide from the assimilatory pathway is incorporated into the O-acetyl serine supplied by serine acetyltransferase (SERAT) to form cysteine as the first stable assimilatory metabolite in sulfate metabolism. This reaction can occur in multicellular compartments including cytosol, plastids, and mitochondria by a pyridoxal phosphate (PLP)- dependent enzyme, O-acetyl serine (thiol) lyase (OAS-TL). OAS-TL plays a coordinating role in regulating the flux between sulfur and nitrogen assimilatory pathways.

The *de novo* biosynthesis of methionine in higher plants occurs in seeds by combining precursors originating from three divergent pathways; (1) O-phospho-homo-serine, a carbon-amino skeleton of aspartate pathway, (2) reduced sulfur moiety from cysteine, an assimilatory product of sulfate metabolism, (3) methyl group from tetrahydrofolate, a one-carbon donor from photorespiration. Hence, the flux through sulfate metabolism is regulated by its metabolic interaction with photosynthesis and photorespiration and indirectly by atmospheric/cellular gaseous composition (CO_2: O_2 ratio) [28].

Complementary strategies to improve seed SAAs

In annuals, seeds are the final storage sinks of nutrient assimilation. Additionally, seeds supply human and animal diet meeting daily nutritional requirements. Although assimilated nitrogen and sulfur are primarily stored as seed proteins, the proteins of cereals and grain legumes are characterized with sub-optimal levels of some EAAs and thereby limit their nutritive value. Cereals are nutritionally deficient in lysine while legumes have an inadequate supply of methionine [33]. These deficiencies in seeds reduce the biological value of proteins to 50–75% compared to a well-balanced amino acid diet thereby leading to protein-energy malnutrition [34]. Further crop plants remarkably vary in the storage form of methionine, for instance, potato tubers store 90% of methionine as a soluble form while, alfalfa leaves and cereal grains incorporate the major methionine into the proteins [35]. Extensive research is required to enhance the sustainable accumulation of SAA in sinks with minimal interference on plant stress response and development. In this context, three complementary approaches to improve sulfur-containing amino acids: genetic engineering, traditional breeding, and agronomy are discussed.

Transgenics based on metabolic engineering

In the past, considerable efforts to manipulate SAA composition in food crops were made through a transgenic push and/or pull strategy (Table 1). It involves genetic modification targeting metabolic engineering of targets at two different levels that enhance the resource capture (push) and storage/remobilization (pull) by expressing the efficient transporter or sulfate assimilatory enzymes or suppressing catabolic enzymes and seed sulfur-rich proteins, respectively. Although genetic engineering of a high-affinity transporter for typical soil sulfate levels is technically feasible, it would be impractical as the activity of transporters are highly responsive to plant nutrient availability. Transporters have evolved to uptake high sulfate under sulfate-deprived conditions and contrarily when sulfate is abundant, uptake is limited. However, this limitation could be offset by expressing the transporters under a constitutive promoter. Nonetheless, the prospects of this approach appear bleak because (1) issues associated with root cell-specific expression of transporter genes and (2) easing of regulatory modules of elemental uptake could lead to over-accumulation of sulfite (SO_2). SO_2 is a potent toxic nucleophile that removes the phytol chain from chlorophyll through sulfitolysis leading to impaired photosynthesis, necrosis, and ultimately affecting plant growth [46]. Although re-oxidizing SO_2 into SO_4 through sulfite oxidase reduces the toxic effect, regulating flux by modifying the metabolic enzyme activity could be the best alternative to improve the capture of available sulfur and enhance the soluble SAA pool [47] (Fig. 3).

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Constitutively expressed the LeST1.1, a high-affinity sulfate transporter of *Lycopersicon esculantum* in Indian mustard (*Brassica juncea* cv. Pusa Jai Kisan) was reported by Abdin et al. [48]. It led to a two-fold increase in sulfate uptake and consecutively enhanced sulfur assimilation into protein, due to higher activity of ATPS, in transgenics grown under both sulfur-sufficient and sulfur-insufficient conditions. Over-expression of *Arabidopsis thaliana AtSAT1* in maize exhibited a 12-fold increase in SAT activity with a concomitant increase of kernel methionine in zein protein fraction by 1.40-fold without impeding growth [39]. Similarly, an ectopic expression of fused transit peptide of *Arabidopsis* rubisco and bacterial EcSAT (*E. coli* Serine Acetyl Transferase) in plastids improved the overall nutrition quality of rice seeds by increasing cysteine, glutathione, isoleucine, soluble, and protein-bound methionine [43]. In contrast, the protein-bound cysteine (37–52%) and methionine content (15–19%) in developing seeds of soybean was increased following the over-expression of ATP sulfurylase, however, the overall crude protein content was hampered in transgenic seeds compared to wild [45]. It was obvious that most of the research was engrossed with seed sulfur metabolism and less attention was paid to source (leaf) sulfur metabolism and then, intermediate transport to sink (seed). Garneau et al. [49] hypothesized that the seed quality

Table 1 Transgenic manipulation of crops to improve sulfur-containing amino acids content

Crop plant	Foreign protein/gene expressed	Impact on SAA	Reference
Soybean	15 kDa δ-zein	Increased cysteine by 15–30% and methionine by 12–20%	[36]
Soybean	18 kDa δ-zein and 27 kDa ɤ-zein	Increased methionine by 27%	[37]
Soybean	Milk bundle protein; MB-16	Increased cysteine by 65.9%, methionine by 16.2% with total SAA content up to 42%	[38]
Maize	*Arabidopsis* Serine acetyltransferase	Increased kernel methionine up to 1.4-fold within 10 kDa δ-zein	[39]
Lupin	Sunflower seed albumin	Increased methionine by 94% and cysteine reduced by 12%	[40]
Common bean	Brazil nut 2 S albumin (BNA)	Increased 12–24% methionine	[41]
Rice	Sunflower seed albumin	Increased 7% total protein with little change in the total sulfur amino acid content	[42]
Rice	*E.coli* Serine acetyltransferase	Increased protein-bound methionine by 4.8fold and free methionine by 2.7fold	[43]
Maize	3′-phosphoadenosine-5′-phosphosulfate reductase (*Ec*PAPR) from *E.coli*	57.6% more methionine accumulation in 10 kDa δ-zein of kernels	[44]
Soybean	ATP sulfurylase	Increased the accumulation of Bowman-Birk protease inhibitor with a raise of protein-bound cysteine and methionine by 37–52% and 15–19% respectively	[45]

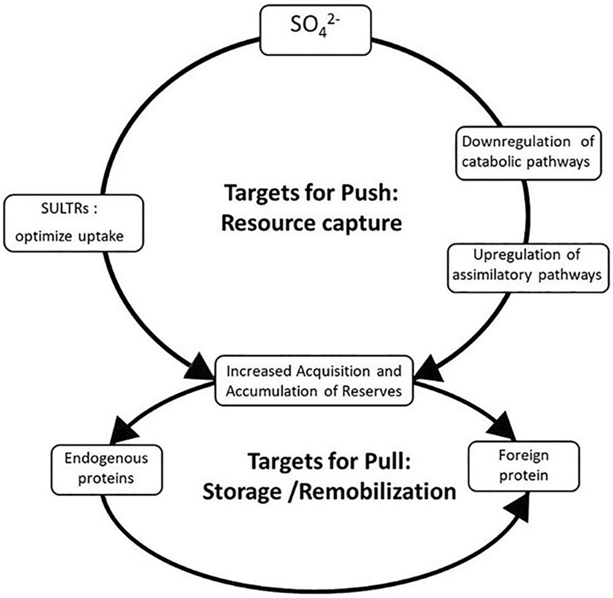

Fig. 3 Schematic representation depicting possible gene manipulation targets to improve the efficiency of sulfate uptake and assimilation in crop plants in response to sulfur: Targets are divided into two levels; Push and Pull. The inter-dependencies of these targets are shown by arrows

of legume crops positively correlated with the amount of organic sulfur allocated by virtue of source-to-sink transport and following its import into developing embryos. Interestingly, RNAi mediated seed specific repression of cystathionine ɤ-synthase (CGS) in *Arabidopsis* led to the synthesis of methionine in seed, but augmented methionine accumulation was found in RNAi seeds compared to control yet with an unknown mechanism [50]. CGS, a key regulatory enzyme supports methionine biosynthesis from phospho-homoserine and cysteine in accordance with the classical aspartate family pathway that exists in both vegetative and reproductive tissues. A ^{13}C -isotopic metabolic-based investigation deciphered that plausible molecular logic of this methionine augmentation is metabolic switching of S-methyl methionine (SMM) cycle in rosette leaves, transportation of SMM through phloem and reconversion back to methionine in seeds. This novel metabolic cross-talk between vegetative tissue and seed involves two types of methyl transferases; methionine S-methyltransferase (MMT) and homocysteine S-methyltransferases (HMT) [32]. Thus, the modulation of the SMM cycle; CGS and MMT activity in leaves, and HMT activity in the seed can be a novel approach to augment the seed methionine pool. A site-specific expression of *methionine Uptake1*, a high affinity met/cys transporter in phloem and seed of *Pisum sativum* L. from yeast improved the allocation of methionine into seed storage proteins (SSP), accounting 23% rise in SSP accumulation with major proportion as sulfur-rich proteins. Further, methionine phloem loading significantly influences sulfate uptake, assimilation, and source-to-sink transport of organic sulfur compounds including SMM and glutathione, and thereby, crop biomass and yield. Thus, it suggests that the role of methionine transporter in source and sink tissue is a critical constraint for allocating sulfur to seeds and its targeted manipulation is imperative for high-quality SSP accumulation [49].

Few legume crops like common bean (*Phaseolus vulgaris*), and few *Vigna* species store a surplus content of sulfur as a part of nonprotein SAA that cannot be reserved as cysteine or methionine in their seeds. The free form of S-methyl cysteine and S-ethenyl cysteine was found high in the early stages of seed development, while in the late stage, they are converted and accumulated as ɤ-glutamyl peptides. In mature seeds, they accounted for up to 0.5% of seed dry weight [51]. With limited information on the biosynthesis of nonprotein SAA in legumes, Joshi et al. [51] hypothesized the probable biosynthetic pathway of S-methylcysteine by using C^{13} labeled amino acid precursor feeding in *P. vulgaris* which is catalyzed by cysteine synthase that condenses OAS and methanethiol. In this pathway, methanethiol is formed by methionine ɤ-lyase. While the biosynthetic pathway of ɤ-glutamyl-S-methylmethionine is yet to be characterized. Additional research can identify candidate genes and enzymes of nonprotein SAA and will provide ample opportunity for improving protein sink.

Certainly, the suppression of genes for endogenous methionine-poor proteins reinforces the synthesis and accumulation of endogenous methionine-rich proteins by proteomic rebalancing. To corroborate this, the suppression of CG-β-1 in soybean through RNA interference under seed-specific oleosin8 promoter resulted in a substitutive rise in the accumulation of sulfur-rich11S glycinin fraction (in particular A2B1 subunit) at the expense of sulfur-poor 7 S conglycinin. Notably, a 1.89-fold change in the 11 S/7S ratio in seed was observed with enhanced SAA content up to 79.19 ηmol/mg (51.38%) compared to the wildtype control. Among 11 S glycinin fractions, A2B1 has a higher proportion of SAA. Further, characterization of the secondary structures of globulin fractions by FTIR, Ramanspectra, and circular dichroism inferred that proteomic rebalance was accompanied by certain conformational transitions having more ordered β-sheets compared to α-helix and β-turn that may improve textural properties [52]. Similarly, an augmented expression of methionine sinks (β and δ-zeins) in maize during endosperm development conversely reduces the cysteine-rich proteins (β- and γ zeins) at the translational level. This inverse amino acid-specific bias and methionine biosynthesis at the expense of cysteine [53] may cause a dearth of kernel vitreousness in quality protein maize (QPM) [54]. Therefore, a balanced amino acid composition in QPM can be achieved by metabolic engineering of both sulfur assimilatory genes and genes for strong sinks [55].

The amount of protein-bound methionine to be accumulated in SSP depends on the total free methionine available for protein accumulation and the number of methionine residues in SSP. To decipher the major regulatory factor of protein-bound methionine accumulation, a sophisticated push and pull strategy was executed to increase the accumulation of methionine-rich sunflower seed albumin storage protein (A1/A2) and the amount of free methionine contents in *Arabidopsis* seeds by transgenic manipulation of SSP and crossing positive transgenic lines with the plant having a high amount of free methionine. An increased accumulation of SSA A1/A2 protein up to 5.3 and 10.5fold was observed in transgenic *Arabidopsis* expressing seed-specific SSA (A1/A2) respectively in comparison to control. Further, the resultant progenies (A1S and A2S) of crosses showed augmented accumulation of protein-bound methionine. However, this change is insignificantly differed from protein-bound methionine in A2 indicating all methionine residues of A2 are saturated with methionine and suggesting that the number of methionine residues in SSP is the major decisive factor for protein-bound methionine accumulation [56]. In second-level targets, the introduction of additional seed-specific sulfur-rich protein as a novel/strong storage sink under seed-specific promoter is a promising strategy to improve the mobilization of the soluble flux of sulfur amino acids into the reserves. The rationality of this approach is that only a fraction of 20–50% soluble methionine gets subsumed into proteins, while the surplus content is eventually transformed into other metabolic derivatives [57]. Thus, modulation of the methionine catabolism in plants warrants trapping the soluble methionine in the storage protein [34]. Unlike nitrogen accumulation, only a few specialized seed storage proteins in endosperm may improve the sulfur sink. The expression of several sulfur-rich foreign proteins including Brazil nut 2 S albumin, zeins, sunflower seed albumin, and MB-16 have been achieved in diverse crop species like soybean [37, 38] lupin [40], common bean [41], rice [42], and potato [58]. Zhang et al. [38] observed that the transcript level of these strong sinks under seed-specific promoters is significantly influenced by the stage of seed development, and decreases in the maturity. Legume B4 promoter, widely used in soybean metabolic engineering, also showed reduced activity in mature seeds. Overexpressing the β-zein gene in soybean with legume B4 seed-specific promoter led to a higher accumulation of foreign protein than with constitutive CaMV 35 S promoter, implying their late-stage synthesis and indicates a consistent scope for efficient inducible promoters in biofortification [59]. Indeed, most of these transgenic events complemented, and protein-bound methionine and cysteine levels were found to be enhanced by 30–97% compared to their non-transgenic counterparts. However, there are many fundamental questions pertaining to (i) the potential allergenicity of the foreign proteins [60] and (ii) the deviation of sulfur flux away from endogenous sulfur-rich proteins because the accumulation of these proteins is restrained by soil sulfur availability [61]. Corroborating the latter argument, Hagan et al. [42] found that transgenic rice expressing sulfur-rich seed albumin has an equivalent level of organic sulfur compared to non-transgenic grain along with an altered composition of sulfur-containing proteins. The expression of storage protein genes is coordinately regulated through transcriptional and post-transcriptional mechanisms and O-acetyl serine is reported to be a central signaling compound in sensing sulfur and nitrogen availability [62]. Hence, a greater comprehension of molecular factors underpinning seed protein metabolism may greatly aid in efficient genetic manipulation of the sulfur assimilatory pathway along with the expression of foreign proteins. It could be a preferred strategy to reduce the negative impacts on endogenous seed reserve remobilization, plant growth, and metabolism [44].

Breeding for cultivars with high-quality proteins

Plant breeding activities are primarily focused on improving crop growth behavior, stress resilience against biotic and abiotic factors, yield, and nutritional improvements including increasing protein content and minimizing anti-nutritional factors. Recently, a trend of biofortification of crops with enhanced mineral nutrient content, protein quality, and composition is gaining importance [63]. The sulfur amino acid content in seed proteins is inherited as a quantitative trait. The utilization of a natural genetic variation for the sulfur amino acid content among germplasm of crop plants due to soil nitrogen/sulfur availability, genetic factors, environmental factors, and their interactions are the basis for biofortification through current breeding approaches. Thus, identifying the candidate genes underlying the sulfur metabolic pathway and understanding the complexity associated with the polygenic trait is mandatory for marker-assisted breeding to improve protein quality [7].

QTLs linked to SAAs are identified and mapped only in a few crops like soybean (*Glycine max* L.), common bean (*Phaseolus vulgaris* L.) utilizing single nucleotide polymorphism (SNP) and simple sequence repeats (SSRs) based genetic markers suggesting insufficient characterization of genetic control of SAA in most food crops. Ma et al. [64] reported a total of twenty-five QTLs associated with Cys, Met, SAA, and protein content spread across four soybean chromosomes (07, 08, 15, and 20) in a recombinant inbred lines (RILs) population (Kefeng No. 1 × Nannong 1138-2). Similarly, Panthee et al. [65, 66] reported an overlap of twelve QTLs related to Cysteine (Satt235, Satt252, Satt427, and Satt436), Met (Satt252, Satt564, and Satt590) glycinin (Satt461, Satt156, and Satt292), and conglycinin (Satt461 and Satt249) in QTL mapping distributed across seven chromosomes (Gm01, 07, 13, 16, 17, 19 and 20). Breeding attempts associated with the selection of either protein yield or protein quality postulated that these traits are negatively correlated. An attempt to improve the protein composition with SAAs resulted in decreased protein content and vice versa [63, 67]. Because SSP genes exist as multigene families, variation in seed protein quality is negatively correlated even after multiple gene manipulation within these families [68]. Thereby limiting the effectiveness of classical breeding efforts. Recombinant inbred lines (RILs), 2–37 and 3–84 have been developed by crossing SSP deficient common bean genotype, SMARC1N-PN1, and a cultivar Modern003. The protein quality of both the lines has improved significantly compared to Modern003, attributed to an increase in total methionine and cysteine contents by 15% and 35%, respectively however total protein content diminished [69]. This variation in SAA content is ascribed to the compensatory accumulation of sulfur-rich proteins to the sulfur-poor SSPs, phaseolin, and lectin through proteomic rebalancing [61]. This postulation was supported by the identification of QTLs among RIL populations, corresponding to phaseolin and lectin loci, and whose deficiency augmented the SAA concentration in sulfur-rich SSPs [69].

The QTL mapping strategy has a major limitation as it depicts the allelic variation of the biparental population only and thus, results in poor resolution. The consequent large confidence intervals among the QTLs limit the effectiveness of QTL maps in the identification of candidate genes [70]. However, these limitations of biparental mapping can be overcome by population-based mapping approaches [71]. Genome-wide association studies (GWAS) is a population-based mapping technique that evaluates the association between each genotyped marker and a phenotype of interest in unrelated individuals, which significantly promotes breeding efforts [72]. Yuan et al. [73] utilized GWAS in unraveling the association of SNPs with sulfur amino acids in a high-density SNP array of 165 soybean genotypes. A strong SNP marker, AX-94,036,794 on chromosome 7 was found to be associated with candidate alleles Glyma.07g175700 and Glyma.07g176000 suggested to be involved in the biosynthesis of amino acids, methionine, and cysteine.

Despite the several reports, QTLs and candidate alleles for SAA, protein content in soybean were partially understood [63, 65, 66, 68]. Further, these traits have to be evaluated for the influence of the environment at multiple locations for several years which severely hampers the development of improved lines [73]. Prolina, R05-1415, R05-1771, N6202, and TN03-350 are a few high-protein soybean lines developed by USDA nonetheless have limited commercial value due to yield constraint, producing a low number of seeds per plant compared to control cultivars [74–77]. It is worth mentioning that 'TN04-5321' is the only commercially developed and released soybean germplasm line with improved SAA containing methionine (3.3% of seed protein dry matter) with no negative impact on yield [75]. To decipher the genetic basis of seed protein quality, the development of family-based, population-based genetic mapping tools and the improvement of protocols for quantitative measurement of methionine and cysteine contents are indispensable. Currently, HPLC and near-infrared (NIR) spectroscopy-based analytical techniques are employed to evaluate the cultivars based on SAA content. However, ineffectiveness in terms of time consumption (HPLC)/precision (NIR) and cost factors limit the application of these techniques in large-scale plant breeding efforts. Rapid and affordable quantification methodologies of cysteine and methionine are crucial for efficient high-throughput screening. It is anticipated that the development of these methods will significantly support soybean breeders in their efforts to create soybean cultivars with improved sulfur amino acid content.

In addition to marker-assisted breeding, plant genome editing approaches could be employed for targeted manipulation of genes to produce transgene-free plants with desirable traits by virtue of sequence-directed nucleases including *ZFNs* (zinc finger nucleases), *TALENs* (transcription activator-like effector nucleases), and CRISPR/Cas (clustered regularly interspaced short palindromic repeats/ CRISPR associated protein) systems. To date, literature highlighting the role of these cutting-edge techniques in sulfur biofortification of crops is lacking, despite their proven potential in metabolic engineering [78]. With the progress in genome sequencing technologies and GWAS, researchers can identify QTLs associated with SAA and sulfur-rich SSP, which will be discussed under the breeding strategy for biofortification. Later, these traits can be invariably employed in high-throughput molecular screening of germplasm, and genome editing tools can recruited to knockout/down-regulate and overexpress the genes involved in catabolic/ secondary sulfur metabolism and primary sulfur assimilation/ endogenous SSP respectively in superior lines.

Seed sulfur regulation and its influence on protein quality

Seed storage proteins are essential sinks of carbon, nitrogen, and sulfur, particularly abundant in endosperm and cotyledons of cereal and legume seeds, respectively. Synthesis of storage proteins is strictly regulated depending upon the developmental stage, tissue specificity, and nutrient availability to plants [79]. Plant sulfur nutrition is vital as plants devote 90% of the sulfur uptake to synthesize sulfur-containing amino acids and are the only primary source of essential amino acids in the diet and feeds. Its optimal uptake is also crucial for plant growth and adaptation to adverse environmental impacts apart from crop production [48]. Hence, the conditions of sulfur deficiency or toxicity affect seed sulfur metabolism as it maintains seed quality and yield [80]. Pandurangan et al. [61] has hypothesized that the reason for the accumulation of sulfur-poor proteins in legumes might be due to their ecological adaptation to soils deficient in sulfur. The dominantly translocated form of sulfur in phloem, sulfate, whose partitioning occurs during the seed-developing stage. Thus, an active partitioning allocates both the reduced (glutathione, S-methyl methionine) and oxidized pools of sulfate into two tissue-specific distinctive pathways i.e., biosynthesis of cysteine and its incorporation into protein in seed embryo and biosynthesis of defense-related sulfur metabolites in seed endosperm and seed coat [81, 82] (Fig. 4). The sulfate assimilation and accumulation in most plants exhibit a similar pattern under sulfur deficiency. Soybean has two prominent seed storage proteins sulfur-rich hexamer protein, 11 S glycinin, and sulfur-poor trimer, 7 S conglycinin. Soybean accumulates sulfur-poor SSPs such as β-conglycinin as a supplement of sulfur-rich SSPs such as glycinin, probably to maintain normal growth and development even under sulfur-deficient conditions [83]. Under these conditions, O-acetyl serine acts in coordination with sulfur-deficiency-induced genes as the master regulator of SSP gene expression in favor of sulfur-poor SSPs [84]. The sulfur content of seed influences the protein quality and yield synergistically with nitrogen metabolism as they are the building blocks of amino acids and also central components of a metabolic enzyme, nitrate reductase (NR). Several studies have demonstrated the potential effect of nitrogen and sulfur nutrition on protein content and composition, respectively [85]. Tao et al. [86] reported that the application of sulfur increases the activities of wheat nitrate reductase and glutamine synthetase in flag leaves, thereby significantly favoring the protein yield, glutenin to gliadin ratio (flour quality), and biosynthesis of SAA-rich proteins. While sulfur deprivation causes the accumulation of high levels of soluble pools of nitrates, amides, and non-sulfur-containing amino acids thereby hampering the biosynthesis of proteins and enzymes due to SAA scarcity [87]. As methionine and cysteine are fundamental to initiate the mRNA translation and formation of protein disulfide bonds, respectively contributing to protein formation and improved stability in consistency to grain protein concentration [88]. On another hand, the storage protein of transgenic lupin seeds with improved methionine may have equivalent or comparably less cysteine content. The absence

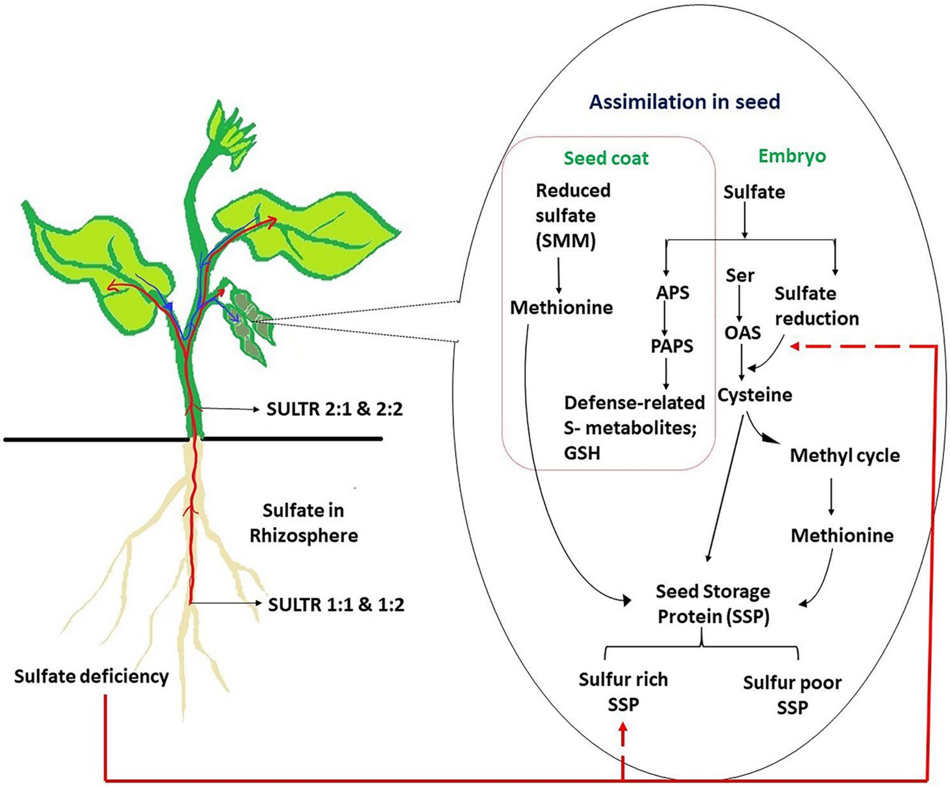

Fig. 4 Schematic representation of sulfate uptake by the plant and seed regulation of seed storage protein synthesis during seed filling stage: Soil sulfate uptake and partitioning in plant is facilitated by SULTRs (red). Reduced sulfate, S-methyl methionine (SMM) is transported into seed coat from other plant parts (blue) to synthesize free methionine. The non-reduced sulfate flux in the seed embryo is partitioned to synthesize free sulfur-containing amino acids and defense-related sulfur metabolites in seed embryo and seed coat, respectively. The flux regulation into these pathways is coordinately regulated by the availability of O-acetyl serine, plant sulfur nutrition. The composition of seed storage protein is under dynamic rebalance depending on the availability of sulfur-containing amino acids, which in turn depends on plant sulfur nutrition

of an additive effect of cysteine (Cys) in the foreign protein, in conjunction with the endogenous seed cysteine, may be attributed to a potential limitation in the availability of sulfur amino acids during the synthesis of storage proteins in developing seeds [62]. This limitation can be overcome by proper fertilizer management. Although the functional sulfate transporters are highly expressed in response to sulfur-deprived conditions, several studies have reported the improvement of protein quality with sulfur fertilization by dynamic proteome rebalance that augments the expression of SAA-rich protein sinks including glycinin in soybean [89] β-ziens and ɤ-zeins in maize [88], 11 S legumin, albumin 2 and defensin D1 in SMARC1N-PN1 genotype of common bean [61], pea albumin 2 and globulins in pea [90, 91]. For instance, in soybean seeds, storage protein accounts for 70%, out of 40% of seed protein, on a dry weight basis. The storage proteins in the soybean are grouped into glycinin (11 S) and β-conglycinin (7 S), each differing in the constitutive sulfur amino acid content of 3-4.5% and less than 1% of amino acid profile, respectively. The low sulfur amino acid profile is correspondingly due to the presence of β-conglycinin fraction in soybean that sparingly contains methionine and only one cysteine per unit protein [92]. However substantial plasticity in the accumulation of seed protein fraction, i.e., increased 11 S to 7 S ratio occurs in response to sulfur nutrition because of downregulation of mRNA encoding for β conglycinin due to increased methionine content [92, 93]. Conversely, sulfur deficiency leads to increased synthesis and accumulation of β conglycinin. A similar influential role of plant nutrition affecting legumin and vicillin was observed in peas [90]. These studies provide evidence of the regulatory impact of sulfur nutrition on the quality of seed proteins when nitrate supply is not a limiting factor. However, during the conditions of nitrogen deficiency, sulfur can impede protein biosynthesis, leading to a decrease in crop yield.

Implementation of machine learning in sulfur biofortification

In recent decades, insights from basic plant research and high throughput omics technologies have greatly expedited crop improvement and strides into the period of high-dimensional biology dealing with large, complex, and heterogeneous multi-omics biological datasets. Unprecedently, the effective integration of these datasets surpasses the capabilities of traditional model-based statistical analyses. In this context, machine learning (ML) algorithms could be an imperative technology due to their greater adaptability and potentiality for integrating extensive, non-structured, and high-dimensional datasets of multi-omics and thereby translating into precision-designed breeding programs [94]. ML belongs to the realm of artificial intelligence that employs statistical, probabilistic, and optimization methods to identify the patterns in existing data, enabling the prediction of novel data points without relying on stringent statistical assumptions. ML has demonstrated a spectrum of applications in biological research [95]. The most commonly used ML algorithms in biosciences are Support vector machine (SVM), artificial neural networks (ANN), random forest (RF), and Bayesian approaches [94]. As stated in this review, storage protein compositions dictate seed protein quality. A detailed comprehension of the structure and diversity of storage proteins is crucial for quality manipulation, revealing the flexibility in altering protein composition without perturbing their biological properties. Therefore, characterizing and classifying these proteins and obtaining typical patterns of storage protein under various conditions like stress and developmental stages of the seed would expedite breeding efforts for enhanced nutritional quality in crop varieties. On the other hand, the SuCComBase database serves as an invaluable for researchers to take forward systemic investigations of SCCs in plants. SuCComBase is a meticulously curated database that reposits all molecular comprehension (genes, proteins, and other related data) related to the biosynthesis of sulfur-containing compounds (SCCs) in plants. By collating data from the literature, a total of 778 probable genes related to SCCs (SCCGs) are identified using 147 known and 92 potential SCCGs in *Arabidopsis* [96]. The identification, and classification of characteristic candidate genes/proteins rely on screening of homologous sequences using computational tools based on identifying sequence/structural similarity and conservation patterns. However, the performance of computational tools like BLAST + and HMMER was found to be laborious and suboptimal in identifying non-homologous sequences [95]. This limitation can be overcome by building models for sulfur metabolism gene/proteins using potential ML approaches, and linking them with storage protein patterns that would advance the research of sulfur biofortification into new milestones.

Development of a model for biological datasets by ML algorithms has three major steps: (1) identification and extraction of a set of features from data, (2) feature selection based on biological indicators to enhance the accuracy of the model and, (3) development of the model. Feature selection is the foremost step in development that removes redundant and noisy features, thereby reducing the time required for data mining and model development [94]. In general, the combination of features such as amino acid composition, length, dipeptide compositions, and physicochemical properties are used as input features for this purpose [97]. In a recent study, a Multilayer perceptron neural network was employed to classify rice SSPs into four classes including Albumins, Globulins, Glutelins, and Prolamins based on molecular weight and conserved functional and structural domains of protein and achieved an accuracy of 95.3% [98]. Similarly, the potentiality of the NM algorithm for the classification of SSPs of five different plants including castor bean, rice, wheat, maize, and thale cress, and its performance compared with other ML algorithms including MLP neural network, decision tree, and support vector machine. A correlation-based feature selection (CFS) algorithm was used to cut down the redundant features from datasets using amino acid composition and sequence length of protein used as input features. An order of high accuracy achieved by NM, wheat (98.6%) > maize (97%) > rice (91.8%) > thale cress (91.3%) > castor bean (82.1%). The measures of model efficacy viz. accuracy, precision, recall, and F-measures found significant in comparison to other algorithms indicated the suitability of the NM algorithm for the classification of SSPs in other crops and determining the quality of different SSPs in larger breeding populations or transgenic crops and screening them to extrapolate in biofortification programs [97]. Similarly, He et al. [95] developed seven ML models for plant SCCGs using SuCComBase, and then an exceptionally performing SVM-Kmer model (F1score = 0.945, ACC = 0.938, AUC = 0.936) utilized to construct an astonishing predictive platform called, 'SCGGs-prediction'. Further, they identified 51,638 SCCGs out of 2,873,697 query protein sequences collected from 81 different species encompassing 69 higher plants and 12 lower plants using this user-friendly platform. The potentiality and robustness of the SCGGs_prediction tool was validated using gene functional enrichment analysis and represented 15 Gene Ontology (GO) terms including glucosinolate biosynthesis, tryptophan catabolism, and indole acetic acid biosynthesis. Further, this algorithm unraveled 501 candidate SCGGs that were previously unidentified by the BLAST + tool and whose KEGG enrichment analysis revealed their significant relation with the SULTR3 family. Undoubtedly, SCGGs_prediction stands out as an indispensable tool for enriching the comprehension of SCGGs with genome sequencing of crop species.

In soybeans, the negative correlation between seed quality traits for seed protein and oil content poses a dire impediment to developing high seed protein lines. The identification of reliable QTLs associated with these traits and possible interaction with other QTLs is a prerequisite for their application in marker-assisted selection. The application of conventional statistical methods such as fixed and random model circulating probability unification (FarmCPU) and mixed linear model for GWAS is limited by large p and small p values that arise due to a larger number of markers than the number of genotypes [99]. Additionally, the application of FarmCPU in GWAS is trammeled due toits incapabilitytofind markers with minor effects and interaction between QTLs, and the significance of markers with major effects on trait and its reproducibility is doubtful and requires repetitive study in several genetic backgrounds and environments. Hence, with the inclusion of its variable parameter, the support vector regression (SVR) mediated ML algorithm in GWAS can sophisticatedly improve the identification of marker-trait association. Yoosefzadeh-Najafabadi et al. [100] identified and localized a varied number of QTLs associated with seed quality traits including seed protein, oil content, and 100 seed weights among 227 soybean genotypes using FarmCPU and SVR-mediated GWAS. SVR-mediated GWAS identified candidate genes associated with QTLs related to 1) seed protein: Glyma.05G186700 (amino acid transport),Glyma.14G035100 (regulate tissue development rely on nitrogen distribution byGlyma.05G186700), Glyma.15G107800 and Glyma.15G109300 (code for protein desumoylation and chloroplast organization respectively, together they harness the energy required for seed protein storage, 2)seed oil content: Glyma.13G187100 encode tocopherol methyltransferase essential for tocopherol biosynthesis and maintain the stability of oil, and Glyma.16G133500 involved in biosynthesis of salicylic acid. Salicylic acid is known to regulate the activity of plant nitrate reductase; hence this candidate gene can be a potential target to alter the negative correlation of oil and protein. Candidate genes associated with 100 seed weight are majorly involved in carbohydrate metabolism-regulating seed size and weight. Hence, identifying QTLs through ML for sulfur metabolism and that associated with seed quality without compromising yield would serve breeders to precisely design breeding for superior varieties with desired traits like seed protein quality, total protein content, and yield.

In conclusion, the genetic and biochemical understanding of sulfate assimilation in plants have progressed well, and the biofortification of SAA has been successful in a few crops like soybean. Nevertheless, much more explorations are required both in the traditional breeding and genetic engineering approaches for achieving this goal. Potential applications of family-based and population-based gene mapping approaches are constrained by efficient and cost-effective high-throughput analytical methods. Genetic engineering approaches should focus on the development of transgenic crops with targeted tissue expression for increased SAA content while minimizing the negative impact on protein yield, plant growth, and productivity. It would be valuable to examine transgenic whole plants under different growth conditions to determine optimal conditions for the SAA level. With the integration of machine algorithms, precise-designed breeding approaches and transgenic manipulation with desired traits can be undertaken and can bridge the gaps in basic research, omics sciences, and translative plant breeding events for sulfur biofortification. Additionally studying the effects of soil nutrient levels and environmental stress on SAA and protein quality would provide important insights to improve SAA.

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