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Review

Soluble Starch Synthase Enzymes in Cereals: An Updated Review

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Abstract: Cereal crops have starch in their endosperm, which has provided calories to humans and livestock since the dawn of civilization to the present day. Starch is one of the important biological factors which is contributing to the yield of cereal crops. Starch is synthesized by different enzymes, but starch structure and amount are mainly determined by the activities of starch synthase enzymes (SS) with the involvement of starch branching enzymes (SBEs) and debranching enzymes (DBEs). Six classes of SSs are found in *Arabidopsis* and are designated as soluble SSI-V, and non-soluble granule bound starch synthase (GBSS). Soluble SSs are important for starch yield considering their role in starch biosynthesis in cereal crops, and the activities of these enzymes determine the structure of starch and the physical properties of starch granules. One of the unique characteristics of starch structure is elongated glucan chains within amylopectin, which is by SSs through interactions with other starch biosynthetic enzymes (SBEs and DBEs). Additionally, soluble SSs also have conserved domains with phosphorylation sites that may be involved in regulating starch metabolism and formation of heteromeric SS complexes. This review presents an overview of soluble SSs in cereal crops and includes their functional and structural characteristics in relation to starch synthesis.

Keywords: amylopectin; cereals; starch synthase; phosphorylation

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1. Introduction

Starch is the primary source of energy for human nutrition and is a main product of plant photosynthetic C fixation [1]. Higher plants synthesize storage starch in the form of granules and store in the seeds and tubers. Starch present in these organs and accumulate during the developments of these organs and its stable for long period of time in dry condition. Most of the starch in seeds store in the endosperm tissue with little amount of starch store in embryo and pericarp. Transitory starch present in leaves of plants and is derived from surplus sugar produced during photosynthesis [2]. Natural sugar which is actually a glucose, development in plants is due to degradation of transitory starch which is transported into the cytosol. Starch plays an essential role in plant physiology and alteration of starch levels affect plant growth, seed yield, and flowering time [3]. The degradation of starch occurs during respiration in plants and contributes to the formation of sucrose. This sucrose is transported to the rest of the plant to provide energy in plant growth [4].

Starch is the major polysaccharide in plants, and is composed of two glucan polymers, amylose, and amylopectin. Amylose is a smaller polymer of α-1,4-linked glucose. While amylopectin is highly branched molecule and major component with α-1,4-linked glucose linear chains and α-1,6-linked branched points. The contribution of amylopectin in starch granule is 75% [5]. Starch is formed from the activated nucleotide diphosphate sugar precursor adenosine-5′-diphosphoglucose (ADP-Glc). ADP-Glc is used for elongation of glucan chains by soluble starch synthase (SS) and non-soluble granule bound SS (GBSS) in amylopectin and amylose synthesis, respectively. These α-1,4-linked glucan chains are branched by the introduction of α-1,6-linked branch points with the coordination of starch branching enzymes (SBE). By trimming at specific points in the nascent granules through starch debranching enzymes (DBE), crystalline starch granules are produced. It is accepted that amylopectin branching frequency and pattern is non-random. These glucan chains are categorized with in each molecule on the basis of their connection to other glucan chains: the external chains that have no branches themselves are A-chains. Similarly, B-chains have one or more clusters (B1, B2, B3). The C chain is the part of B-chain in a molecule with free reducing end. The frequency distribution of chain length shows that mostly chains consist of 20–30 glucose units and these are A- and B1-chains in the cluster of amylopectin [6] (Figure 1). Similarly, there are protein targeting to starch (PTST) enzymes (PTST2 and PTST3) which take part in granule initiation in plants and loss of these enzymes causes reduced number of granules in chloroplast. In plants, SSs are GT-B-fold glycosyltransferases, classified within family GT5 in the CAZy database. The archaeal and bacterial GS are the closest counterparts of plant SSs in the GT5 family [7], implying that this family is ancient. All of them use ADP-glucose as nucleotide donor sugar. However, GS in other eukaryotes, such as fungi, yeast and animals, are distantly related to plant SSs, and belong to the GT3 family in the CAZy classification, using UDP-glucose as donor [8].

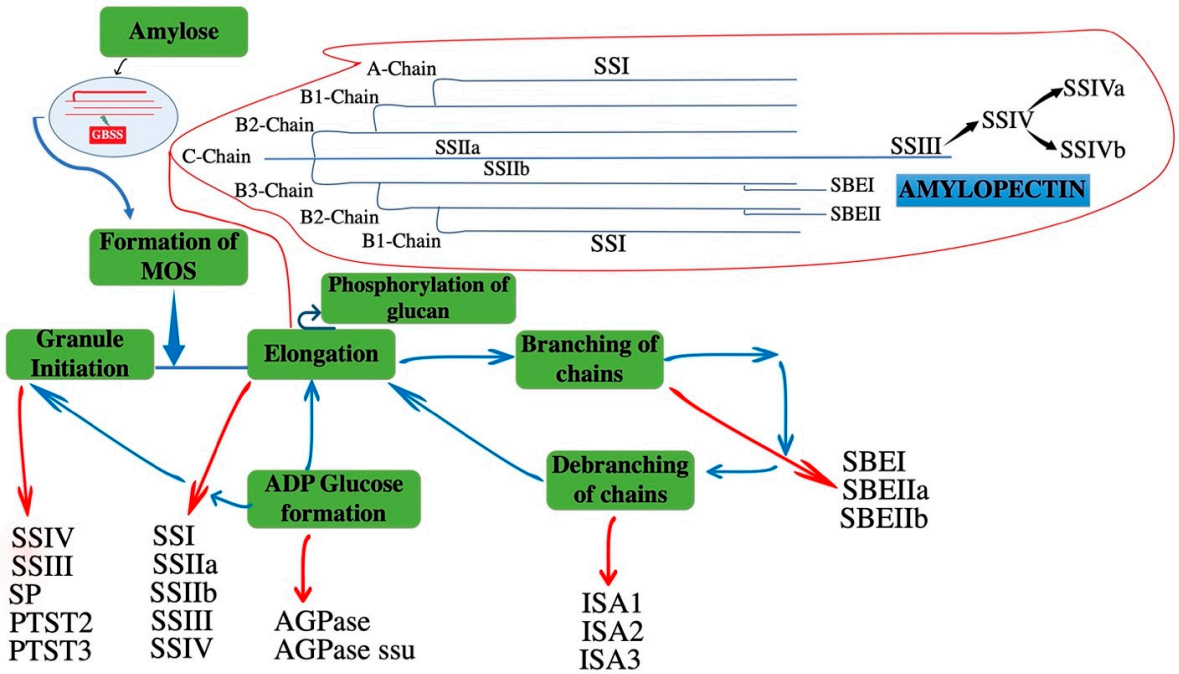

Figure 1. A schematic of enzyme-mediated reactions involved in the formation of starch, amylose, and amylopectin. The diagram represents the interconnection of non-linear reactions of different enzymes during starch biosynthesis. Each class is highlighted with an arrow that is showing each stage of starch biosynthesis with different enzymes. The red arrows mentioned the enzymes of different stages and blue arrows mentioned the relation of different stages during starch formation. ADPglucose pyrophosphorylase: AGPase; ADPglucose pyrophosphorylase small subunit: AGPase ssu; Isoamylase-type debranching enzyme 1, 2, 3: ISA1, ISA2, ISA3; Starch synthase I, IIa, IIb, III, IV: SSI, SSIIa, SSIIb, SSIII, SSIV; Protein targeting to starch: PTST2, PTST2; Starch phosphorylase: SP; Starch branching enzyme I, IIa, IIb: SBEI, SBEIIa, SBEIIb; Granule bound starch synthase: GBSS.

Most of the enzyme classes described have multiple isoforms with overlapping functions [9]. Soluble SSs (SSI, SSII, SSIII, and SSIV) function in the process of starch synthesis have been elucidated by mutant analysis of monocots by using cereal models and of dicots through studying potato tubers, *Arabidopsis* leaves, and pea embryos. In *Arabidopsis*, it regulates the granules numbers that form in the chloroplast and it is closely related to SSIV. SSV is a noncanonical isoform with no catalytic glycotransferase activity [10]. The structure and size of amylopectin clusters are mainly controlled by three soluble SSs (SSI, SSII, and SSIII), with the interconnection of SBE and DBE enzymes (Figure 1) [11]. Many SSs genes are present in cereal crops, and their copy number is different in each cereal, presumably reflecting gene duplication, deletion, and genomic polyploidization during evolution (Table 1).

Table 1. Starch synthase genes in cereal crops.

Species	No. of SS Genes	Gene Names with Accession No./ID	Reference
Hordeum vulgare	6	*GBSSI (AAM560327.2), SSI (AAF37876), SSII (AAN28307), SSIIIa (AAF87999), SSIIIb (AAL40942), SSIV (AAK97773)*	[12]
Oryza sativa	11	*GBSSI (AB425323), GBSSII (AY069940), SSI (AY299404), SSIIa (AF419099), SSIIb (AF395537), SSIIc (AF383878), SSIIIa (AY100469), SSIIIb (AF432915), SSIVa (AY373257), SSIVb (AY373258), SSV (EU621837.1)*	[13]
Sorghum bicolor	10	*GBSSI (LOC8068390), GBSSII, SSI (NC054143), SSIIa (EU620718), SSIIb (EU620719), SSIIIa (EU620720), SSIIIb (EU620721), SSIV, SSV (HQ661801)*	[14]
Triticum aestivum	7	*GBSSI (AF286320), GBSSII (AF109395), SSI (AJ269503), SSII (AJ269503), SSIIIa (AF258608), SSIIIb (EU333946), SSIV (AY044844)*	[15]
Zea mays	10	*GBSSI (AY109531), GBSSII (EF471312), SSI (AF036891), SSIIa (AF019296), SSIIb (EF472249), SSIIc (EU284113), SSIIIa (AF023159), SSIIIb (EF472250), SSIV (EU599036), SSV (NM_001 130131.1)*	[16]

In this review, we provide an overview of soluble SSs and its roles in starch biosynthesis. The purpose of different isoforms in cereals will also be discussed about studying different mutants. The current knowledge of SSs regulation, their ability to form protein complexes with other enzymes, and their regulation by protein phosphorylation are outlined.

2. Mode of Action and Properties of Soluble SSs in Amylopectin Formation

For the elongation of the α-glucan chain during amylopectin synthesis, three enzymes (SSI, SSII, and SSIII) play important role (Figure 1). Similarly, SSIV is involved in granule initiation and shows close relation to SSIII [17]. SSI, SSII, and SSIII elongate α-glucan chains during amylopectin synthesis with increasingly higher DP (degree of polymerization). SSI synthesizes α-glucan chains from short to intermediate sizes of DP8-12, which are then used as the substrate of SSII to manufacture longer chains of DP12–30. Similarly, SSIII produces long chains of DP $\geq$ 30 [18]. The products and substrate of these SS isoforms are generalized, and it is inferred from the data of the mutant studies in monocots, dicots, and also upon in vitro biochemical analysis [19]. These three isoforms play an essential role in defining the structure of amylopectin by cooperating with SBEs and DBEs [11] (Figure 1). Although there are variations in glucan chains of different species, the glucan chains found in amylopectin clusters are characteristically of short to medium length appropriate for SSII activity [20]. The binding ability of SSI increases dramatically with the length of substrate chains and is inversely proportional to the catalytic capability of an enzyme. SSIII is thought to be involved in connecting amylopectin clusters because organisms lacking SSIII showed a significant reduction in length of cluster-spanning B chains (B2–3) [21].

Tissue-specific isoforms of SSII and SSIII are present in cereals. These isoforms are thought to be involved in long- or short-chain starch synthesis in different heterotrophic

and autotrophic cell types (Table 1) [22]. It is believed that structural variation between starches from different sources is due to the relative contribution of each SS class in various tissues and among species [23]. Due to action of multiple enzymes and alteration of biosynthetic pathways help to cause these structural variations.

2.1. SS Mutants Vital Roles in the Formation Amylopectin Chains: Starch Synthase SSI to SSIII

Loss of SSI activity causes distinct variation in chain length distribution of amylopectin, particularly in A- and B1 chains that help to construct amylopectin clusters. Amylopectin from the endosperm of a *ssI* rice mutant have shorter chains with DPs of 6–7, while there were fewer chains of DP 8-12 [24]. Similar results were observed in *Arabidopsis* mutant *ssI* [25]. In maize, rice, and wheat, *ssI* mutants possessed short chains with DP < 10 (Preiss, 2018). These findings suggest that SSI elongates short chains, mostly DP 8-10, with SBE through their glucanotransferase reactions and create the short chains on which SSI is thought to be act [6]. It is interesting to note that the absence of SSI prevented the formation of short chains but elongated further chains with DP18. By using modified glycogen substrates, it is reported that the N-terminal mutant of maize for SSI drastically decreased the external chain length, but sharply increased SSI substrate binding ability [26]. The evidence suggested that the localization of SSI depend on starch binding by its interacting partner SSII [27]. However, it is unclear why there are short chains in wild type (WT) generated by SSI that were not extended further. A complete deficiency of SSI and SSIII in double mutant (*ssI/ssIIIa*) caused male sterility with opaque seeds in rice [28]. Similarly, the absence of SSI and BEIIb (branching enzyme IIb) leads to male sterility in *japonica* rice and this double mutant had reduced SSI level [29].

The mutant of *SSII* has been characterized in different crops to understand its function such as in potato tubers [18], cereal crops (endosperms of wheat [30], barley [31], rice [24], maize [32]), and in *Arabidopsis* leaves [33]. The observed phenotype in all crops is similar and indicates a significant change in amylopectin structure. The chain lengths of DP8 and DP18 increased and decreased in such mutants, respectively. Similarly, *ssII* mutants had changes in granule morphology accompanied by high amylose content and reduction in starch crystallinity [6]. Mutation for *SSIIa* in barley, rice and wheat have similar effects on starch structure and the amylose content but the difference in the severity of phenotypes. *ssIIa* mutant in rice, wheat and barley altered the structure of amylopectin which deprive the affinity of *SSI* to amylopectin [34]. In cereal crops, SSIIa interacts with SSI and BEII [35]. So, there can be pleiotropic effects on these enzymes due to the misfunction of SSII, making it difficult to understand the impact on phenotype due to the absence of SSII activity alone. Similarly, changes in amylopectin structure are caused by a lack of SSII activity [6]. Firstly, the recombinant rice SSII was incubated with amylopectin from the mutant *ssII*, which was able to promote aberrant elongation of the short chains [36]. Secondly, there was a loss of SSI activity in the *ssII* mutant, which caused typical ssI-type alterations in the background [37]. Thirdly, there were similar changes in amylopectin chain length distribution (CLD) in dicot plants while there is not any evidence in the formation of SSII-containing complexes [6]. The repression line for *SSIIa* and *SSIIIa* showed chalky grain appear and increased in amylose content and also decreased in viscosity in rice. In the amylopectin, there was reduction in short and long chains in grains, but number of medium chains increased. This genetically modified line nature depicted that these two genes interact each other [36].

The function of *SSIII* is less clear as compared to *SSI* and *SSII*. The primary role for SSIII is the formation of the B chain, elongation of cluster filling chains, and regulation of other starch synthesis enzymes. Similarly, it is also reported that SSIII also takes charge of granule initiation in the absence of SSIV. SSIII has significant activity in all plants and tissues. Analyses of *ssIII* mutants of maize [38] and rice [39] revealed that fewer long cluster spanning B chains (such as B2, B3, etc.) were present in mutant lines. There was also alteration in the short chains of amylopectin, indicating that SSIII also participates in the synthesis of short A and B chains [40]. These results were confirmed when compared to

plants that lack SSI or SSII. It was also reported that the absence of SSII significantly affects SSIII, which results changing in the phenotypes of rice [36] and *Arabidopsis* [41] leaves of mutant *ssII* lines, suggesting partial loss of function between these two genes. The *ssII/ssIII* double mutant produced shorten chains with a low number of water-soluble glucans in Arabidopsis [42]. Similarly, loss of SSIIIa caused slightly reduction starch content with little rounded and smaller shape of granules in rice [43]. Additionally, the expression level of granule-bound starch synthase I (GBSSI) and ADP-glucose pyrophosphorylases increased due to absence of SSIIIa which increase amylose content [43], some of the cereal crops ss mutants are described in Table 2.

Table 2. Mutation effect on starch synthase genes in different cereal crops.

Cereals	Amylose Content (%)	Inactivated Genes	Mutant Lines	Structural and Functional Changes in Mutant	Reference
Wheat	22.9–32.3	*SSSII*	*sgp-1*	Alteration in amylopectin structure, high amylose contents	[44]
		SSII	*sgp-1*, *a7*, *a63*	Increase in short chains, decrease in starch branching enzyme	[45]
		SSIIa		Increase in proportion of short chains, difference in gelatinization, retrogradation and pasting	[46]
		SSIIa	*svevo*, *semolina*	Increased in dietary fiber of contents, change in total starch content, improved quality traits	[47]
		SSIIa	*ssIIa-Ab*	Amylose contents increased 3%, cooked noodles firmness increased	[48]
		SSIIa, *GBSS*	*sw*	Changes in seed size, starch granules and starch content, shrunken seed during maturity	[49]
		SSIIa	*abd null line*	Grain properties (change in 1000 grain weight, grain size) and starch properties (fluctuation in amylose content, increased in resistant content) changed in null line	[50]
		SSIV-D	*e054-13*, *e1137*	Altered granule number/chloroplast	[51]
		SSIV	*e3-1-3*, *e1137*	Total starch and amylopectin content decreased	[52]
Rice	15.4–25	*SSI*	*e7*, *i2-1*, *i2-2*, *i4*	Decrease in chains with DP 8 to 12, Increase in chains with DP 6 to 7	[26]
		SSI, *SSIIIa*	*np*	Higher amylose content, internal chain length of B2 and B3 fractions observed	[24]
		SSI	*ss1*, *isa1*	Take part in chain length distribution, outer chain elongation with little effect on branch position distribution	[53]
		SSI, *BEI*	*ss1/be1*, *ss1/be2b*	Seed weight of mutant was higher than WT Number of short chains of amylopectin decrease, Amylose content almost same to WT	[54]
		SSI	*ssI*, *be2b*	Subtle difference in protein profile, reduced association of SSI and BEIIb in *ssI* mutant	[55]
		SSI, *SSIIa*, *SSIIIa*	$ss1^{L}/ss2a^{L}/ss3a$	Increase amylose, decrease grain weight, increase in level of ADP-glucose pyrophosphorylases	[56]
		SSII	*zhonghua-15*	GC-AG intron splicing offer more variants for genetic divergence in rice	[37]
		SSIIa	*ss2a(em204)*	SSIIa protein was totally absent in seeds, higher amylose content, Number of short chains formation increased in amylopectin	[57]
		SSIIIa	*ss3a-1*, *ss3a-2*	Chains with DP 6 to 9 and DP 16 to 19 decreased, chains with DP 10 to 15 and DP 20 to 25 increased, amylose and amylopectin content increased	[58]
		SSIV-2	*allelic variation*	Affected gel consistency, percent of retrogradation,	[59]
		SSIIIa	*flo5-1*, *flo5-2*	Starch granules smaller and round as compared to WT, reduced contents of long chains	[60]

Table 2. *Cont.*

Cereals	Amylose Content (%)	Inactivated Genes	Mutant Lines	Structural and Functional Changes in Mutant	Reference
		SSIIIa, *SSIVb*	*ss3a*, *ss4b*	Produced compound type starch granules in the early stages, glucan chain length distribution identified overlapping roles for SSIIIa and *SSIVb* in amylopectin chain synthesis	[61]
		SSIVb		Transgenic plant contains premature codons, no mRNA expression, low starch contents, dwarf phenotype	[62]
Maize	25–30	*SSIII*	*dull1*	Lager clusters of chain with more branched building blocks, average cluster contained 5.4 blocks in mutant and 4.2 blocks in WT.	[63]
		SSIII, ISA2	*du1-R4059*	Starch deficient, accumulation of phytoglycogen	[21]
		SSIIa	*sugary-2*	Loss of activity of endosperm specific SS, impact on the *SSI* and *SBEIIb*	[64]
		SSIII	*w64a*	Reduced granule size, decreased the enthalpy change of starch gelatinization	[65]
Barley	29.9–31.6	*SSII*	*m292*, *m342*	Decrease in amylopectin synthesis, pleiotropic effect on other enzymes of starch biosynthesis	[66]
		GBSS, *ISA1*, *SSIIa*	*Sex6*, *wax*, *lys5fisa1*	*SSIIa* mutation caused low seed weight and starch content	[67]
		SSI, *SSIIa*, *GBSS*	*TILLING*	*SSI* mutant increased A and B granules, *SSIIa* mutant caused shrunken seed	[31]

SSI: starch synthase I; SSII: starch synthase II; SSIII: starch synthase III; SSIV: starch synthase IV; GBSS: granule bound starch synthase; BEI: branching enzyme I; ISA1: isoamylase-type debranching enzyme 1; TILLING: Targeted induce local lesion in genomes.

2.2. Initiation of Starch Granule Formation

SSIV is involved in the initiation of the starch granule. It controls the number of starch granules in the leaves of *Arabidopsis*, which shows that its function is unique from other genes of the *SS* family [41]. The high level of starch accumulated in potato leaves is gained by a dramatic increase in the expression of the *SSIV* [68]. The presence of SSIV in the thylakoid membrane suggests that starch granule initiation occurs at a specific area of the chloroplast. Gene structure analysis revealed that exon and intron structure of *SSIII* and *SSIV* are highly conserved in *Arabidopsis*, rice, and wheat while gene structure is different from *SSI*, *SSII*, and *GBSS* [69].

The *Arabidopsis ss4* mutant plant showed a reduction in starch granule number but had enlarged starch granules. In this case, the ADP-Glc pool is likely allocated to fewer starch granules thereby leading to considerably larger granule size in the mutant in comparison to WT (Columbia-0) plants [41]. So, this is a clear indication that the initiation of starch granules at least partially requires SSIV in Arabidopsis leaves. Interestingly, the *ssI/ssII/ssIII* triple mutant of Arabidopsis was able to form normal granules in the chloroplast with less starch content, highlighting the function of SSIV because granules numbers were normal in triple mutant plant [70]. Recent studies showed that Arabidopsis plants lacking SSIII and SSIV showed no starch granule formation. Overexpression of SSIV increased the level of starch accumulated in the leaves of *Arabidopsis* by 30–40% and caused a higher rate of growth. This overexpression of *SSIV* did not drastically affect other genes and only slightly altered the expression of *APS1* and *SSIII* [71]. The leading role of SSIV is to coordinate starch metabolism during leaf expansion and to determine the flattened discoid shape of starch granules [72]. It is depicted that the transcriptional regulation of starch synthesis varies among all *SS*. The variation is minor, but the differences are more prominent for *AtSSI* and *AtSSIV* [71]. A strict correlation between promoter/gene sequences and transcription level indicates that *AtSSIV* is subject to *cis*- regulation, while

the absence of this correlation in the other *SS* genes shows that they have a *trans*-regulatory mechanism [73].

Observations from reverse transcription PCR, western blotting, or zymograms indicate that in rice, *OsSSIVa* is mainly expressed in the endosperm and *OsSSIVb* expresses in leaves as well as other developmental stages [74]. It was also observed that the initiation of starch granule synthesis in rice endosperm does not solely depend on *OsSSIVa* and *OsSSIVb* because suppression of these genes does not inhibit the granule initiation process [61]. Strong expression of these genes is observed in sink leaves but is low in seeds. There was weak interaction between SSIV and SP (starch phosphorylase) in maize during protein-protein interaction [35]. *TaSSIV* is expressed in leaves and seeds of wheat [75]. The identification of differences at the amino acid level in TaSSIVb and OsSSIVb in their glycosyltransferase domains might indicate different functional associations [76,77]. *TaSSIV* overexpression increases the accumulation of starch in both photosynthetic and sinks organs. Considering all the limitations inherent in basing conclusions on genetically engineered plants, the results have shown that overexpression of SSIV helps to increase starch content in different autotrophic and heterotrophic organs [78]. However, *TaSSIV* importance related to granule formation remains unknown. Firstly, it is also not known in wheat whether *TaSSIV* is essential for maintaining the starch granule number in mature leaves, or if it has a role in immature leaves where new granules arise during chloroplast division [70]. Secondly, it is also not known if a reduction in starch granule number in the *TassIV* mutant is due to the direct or indirect consequence of the loss of *TaSSIV* because mutants have additional pleiotropic phenotypes in which altered granule anatomy and morphology reduce plant growth as a result of mutation [78]. Third, it is also suggested that overexpression of this gene results in a higher concentration of starch at the end of the day and accelerated plant growth [75].

Among the starch synthases, SSV is most closely related to SSIV, a major determinant of granule initiation and morphology [79]. However, unlike SSIV and the other starch synthases, SSV is a noncanonical isoform that lacks catalytic glycosyltransferase activity. Nevertheless, loss of SSV reduces starch granule numbers that form per chloroplast in Arabidopsis, and ss5 mutant starch granules are larger than wild-type granules. Like SS4, SS5 has a conserved putative surface binding site for glucans and interacts with MYOSIN-RESEMBLING CHLOROPLAST PROTEIN, a proposed structural protein influential in starch granule initiation [10].

2.3. Impact of Different Mutation Technologies on Soluble SSs Genes

In the basic research of soluble starch synthase enzymes, great progress has been done by increasing starch content in different cereal crops. High starch content with improved good quality varieties have been developed in many cereal crops (wheat, maize and rice) by using different mutation technologies such as TILLING, TILLING by sequencing, cloning mutant alleles causative for improved traits and various genetic manipulation in starch synthase genes through different chemicals (ethyl methane sulphonate) and gamma rays.

The effective methods to study starch synthase enzymes are induced mutation and TILLING to generate point mutation. This reverse genetic strategy is suitable for all cereal crops which can be used to detect the functional SNPs from the mutant population and help to evaluate desired traits. SSIV gene had been studied to identify functional mutation through TILLING and understanding the function of this gene [51]. Similarly, the missense mutation for SSI in barley increased the proportion of A and B granules and nonsense mutation for SSIIa change the proportion of amylose/amylopectin ratio and reduced the size of A granules [27]. Marker assisted selection is also effective method for understanding soluble starch synthase and help to fluctuate the amylopectin concentration. Functional markers were developed in SSIV and these markers were used to screen the Chinese wheat population. This functional marker showed significant association with thousand grain weight [69].

3. Structure-Function Relationships of SS in Cereals

Cereals possess multiple isoforms of SS, which are categorized based on conserved amino acid sequence relationships (Table 1). SS has a highly conserved C-terminal motif in which there is a conserved motif of K-X-G-G-L which is responsible for substrate binding [19]. While at the N-terminus of SS, there is variation in length and amino acid sequence which might define the function of each SS isoform. These SS isoforms are highly conserved in higher plants (dicots and monocots) [80]. The sequence of encoded proteins shows a high degree of similarity, but their expression is different among specific isoforms, seemingly divided into predominate expression in vegetative parts or in the endosperm [19] (Wang et al., 2015). Furthermore, there are several isoforms in cereals in each class of SS except for SSI and SSV. For example, SSIIa and SSIIIa isoforms are mainly expressed in the endosperm [81]. Some species have one isoform in each class, such as in *Arabidopsis* and potato.

A phylogenetic tree was constructed to explore the evolutionary relationship of SS in cereal crops (Figure 2). Our analysis depicted that SS isoforms in cereal crops have experienced gene duplication events to different degrees and SSIV showed a close relationship with SSIII, which indicated that their functions are similar in cereal crops [82]. Similarly, SSI showed a close relationship with SSII. Based on protein sequences, it is believed that SSs belongs to glycosyltransferases (GTs) domains [83]. According to domain analysis in cereal crops, it has been detected that GT1 and GT5 domains are present in almost all SS isoforms except for SSV in which there is just a GT5 domain (Figure 3). The primary function of these domains is to catalyze the transfer of glucose to the non-reducing end of the already existing glucosyl acceptor chain to form the α-1,4-glycosidic bond to elongate the chain [58]. In barley SSI and rice GBSS, the catalytic domain has a GT-D fold, which has an active site in the cleft between these two domains (GT1 and GT5) [79].

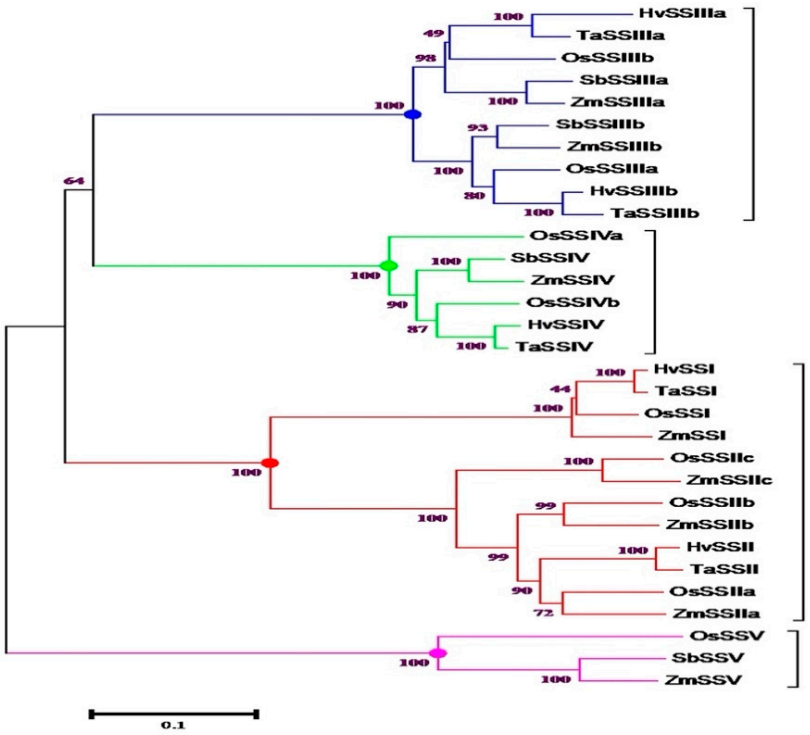

Figure 2. Phylogenetic tree showing the relationship between starch synthase enzymes (SSs) in different cereal crops. The tree was designed by the neighbor-joining method. The bootstrap scores higher than 40 are shown here. Each node is labeled with the prefix of the respective species. The protein accessions numbers are given in Table S1. HvSSI, II, III, IV: Hordeum vulgare starch synthase I, II, III, IV; TaSSI, II, III, IV: Triticum aestivum starch synthase I, II, III, IV; OsSSI, II, III, IV, V: Oryza sativa starch synthase I, II, III, IV, V; ZmSSI, II, III, IV, V: Zea mays Starch synthase I, II, III, IV, V.

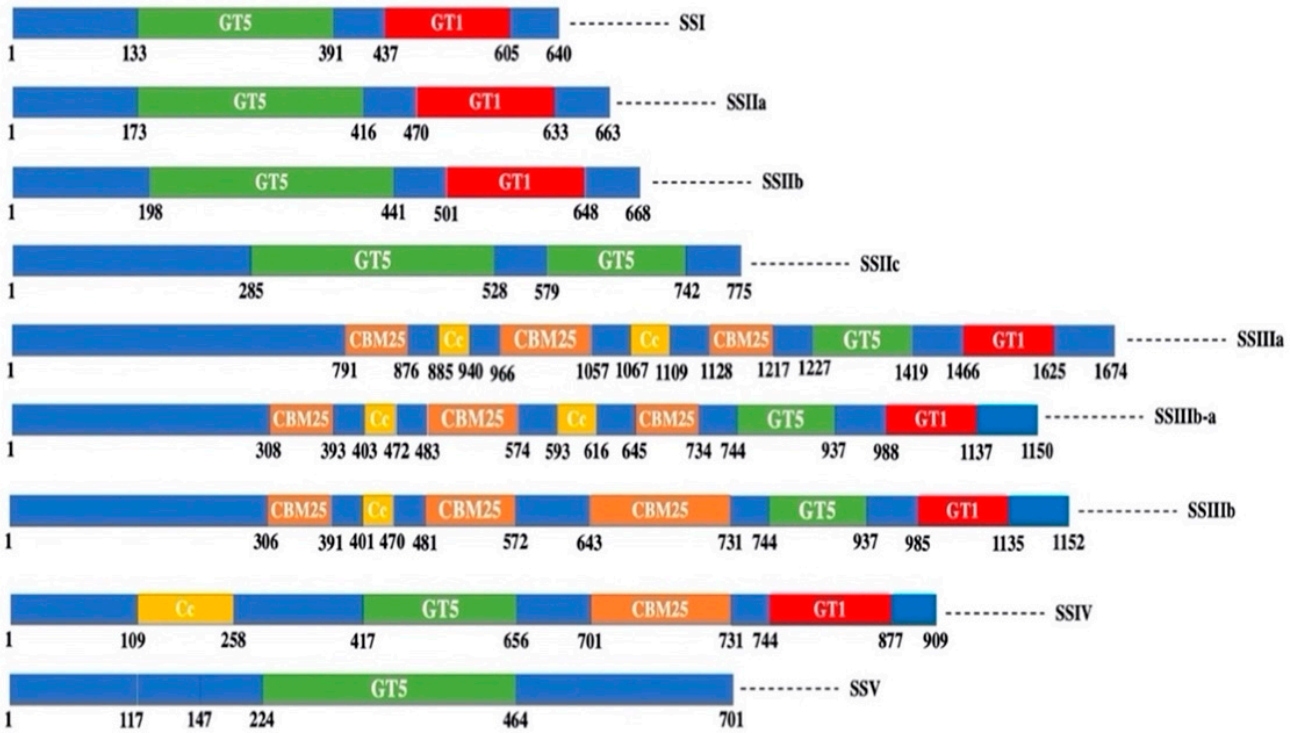

Figure 3. Domain structural features and active site of SS proteins. *Zea mays* SSs are shown as an example. The distribution and composition of domain structures are marked. The N terminus is on the left side of the figure and C terminus is on the right side. The glycosyltransferase family 1 (GT1) and glycosyltransferase family 5 (GT5) domains are shown in red and green colors, respectively. Conserved carbohydrate binding modules of family 25 (CBM25) are represented by orange coloration and coil coiled domains (Cc) are in yellow.

There are either one or two coiled-coil (Cc) motifs in SSIII, SSIV, and SSV in the N-terminal region, which play an essential role in protein-protein interactions [84]. In SSIII, three conserved binding modules (CBM-25) are detected in the N-terminal region and play an important role in substrate binding [85]. The secondary structure of SS isoforms of maize was developed on the basis of the reference model of SSI in wheat, which showed 83% similarity to maize SSI [86]. In maize, this secondary structure analysis showed the difference between SSI and SSII in the GT5 domain and both of these are different from SSIII based on the composition and position of β sheets and α helices. Similarly, the main difference between SSIV and SSV was in the GT1 domain due to missing α helices in SSIV (Figure 3) [82]. In SSV, the active site of this isoform is less conserved, but there is a small portion that showed similarity to SSIII and SSIV (Figure 3) [87]. After studying the analysis of the motifs, it was revealed that the motif "24" is only present in the SSII isoforms. Apart from motifs "20" and "24," the composition of SS and GBSS motifs are similar. In SSIII, unique motifs were identified which are present in GT1 and GT5 domains represented as motif "1" and motif "26" and these motifs are totally unique from other SS motifs (Figures 3 and 4) [82]. In SSs N-terminal regions of the GT-5 domain possesses a highly conserved KXGGL motif "1" and this motif is very well conserved between the different SSs and play very important role in the binding of substrate. Similarly, all SSs contain motif "VIII" towards their C-terminal and it is known as "KTGGL look like". This motif is less conserved between the different SSs [82].

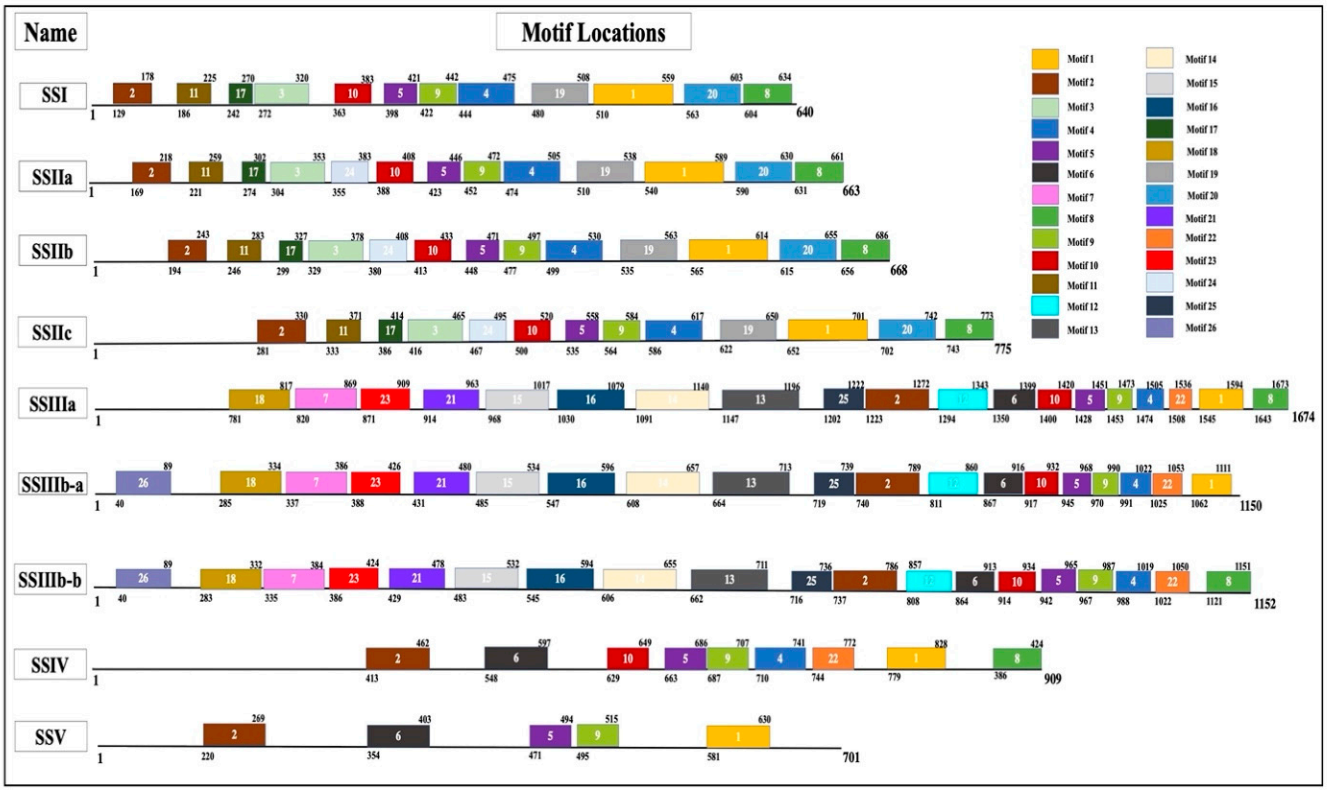

Figure 4. Conserved motifs of Starch synthase proteins. *Zea mays* motifs are shown as example. All sequences were analyzed through MEME to discover conserved patterns.

Similarly, the exact role of the other motifs needs to be resolved in SSs, but it can be said that these motifs may be involved in the formation of three-dimensional structure and have contribution in the ADP-binding and domain stability. The GT-1 domain possesses the number of conserved motifs which have been found in a functionally heterogenous group of glycosyl transferases [82].

4. Regulation of SS Activity in Cereals

Each class of SS plays a distinct role in amylopectin synthesis. As previously described, A- and B1- chains are mainly formed from SSI and SSII isoforms to synthesize short cluster-filling chains, while SSIIIs form long cluster-spanning chains (B2–3 chains) [22]. Similarly, SSIVs are not involved in amylopectin chain elongation but are involved in granule initiation and control granule morphology [52]. In fact, the situation is more complicated when we consider the overlapping functions between different isoforms, the involvement of starch degrading enzymes, and the formation of enzyme complexes [17]. There is also a sizeable gap between our understanding of how each class is regulated at the molecular level.

The contribution of each SS varies in different tissues and between different species, which gives us the idea that there is structural variation in starches from various sources. SSI and SSIII play an essential role in maize endosperm through soluble SS activity, as *SSI* showed no transcript in the leaves of maize [88].

In *Arabidopsis* leaves, the dominant soluble SS appears to be SSI judging by *AtssI* mutant analysis, followed by SSIII, and finally SSII [71]. SSIV contributes little to total SS activity, even though the expression of this enzyme is reasonably high [89,90]. However, the contribution of each SS is difficult to elucidate due to complex genetic interactions. For example, suppression of *SSIII* caused upregulation of *GBSS* and *SSI*, thereby changing the background SS activity [82]. Furthermore, it has also been estimated that SS assays themselves might be better suited to measure the activity of one SS class over another, thus

making activity estimations inaccurate. The mutant activity in SSIV showed a reduction in function of granule formation, only explained in rice [36] and *Arabidopsis* [91].

4.1. Regulation of Protein Phosphorylation

The research related starch phosphorylation was started in the early 20th century by detecting the small amount monoesterified phosphate in the potato starch. Phosphorylation helps to improve the physiochemical properties of starch. Phosphorylation amount varies based on organ of plant and species [92]. Starch biosynthetic protein complexes were initially identified in the endosperm of wheat and were later found in wheat leaves [30]. By using endosperm of other cereal crops, further evidence was collected [91] (Wu et al., 2016). Phosphorylation-dependent complexes with members such as SSI, SSIIa, BEIIa, and BEIIb were identified in barley endosperm [93]. In rice endosperm, gel filtration analysis revealed that SSIIa, SSIIIa, SSIVb, BEI, and BEII formed a high molecular weight protein complex and this protein complex larger than those found in maize. Starch biosynthetic protein complexes are present in the endosperm of cereal crops but still there is need to explore these complexes in the non-cereal crops. It is not compulsory that phosphorylation sites are conserved in all plant species even though phosphorylation-dependent protein complexes are present in wheat, maize, rice, and barley [94]. For starch granules, *SSI*, *SSII*, and *BEIIb* must play a role in starch synthesis as a maize mutant *ssII* had undetectable levels of *SSI* and *BEIIb* in starch granules [64]. This phenomenon was also observed in barley and rice *ssII* mutants [94]. However, the relationship between SSII activities and the phosphorylation-dependent formation of protein-protein complexes remain obscure [64].

In wheat, there is need to study SSII phosphorylation sites for the formation of protein complex. The protein sequence alignment of different species such as Arabidopsis, rice, wheat maize and barley indicated that only Thr323 is highly conserved site. This conserved site is not present in wild wheat, but it is present in the waxy wheat [95]. The phosphorylation sites of SSIIa of wheat are detailed in Figure 5. The impacts of putative phosphorylation sites need to be verified using biochemical and mutant analyses to understand their role in starch biosynthesis and complex formation [96].

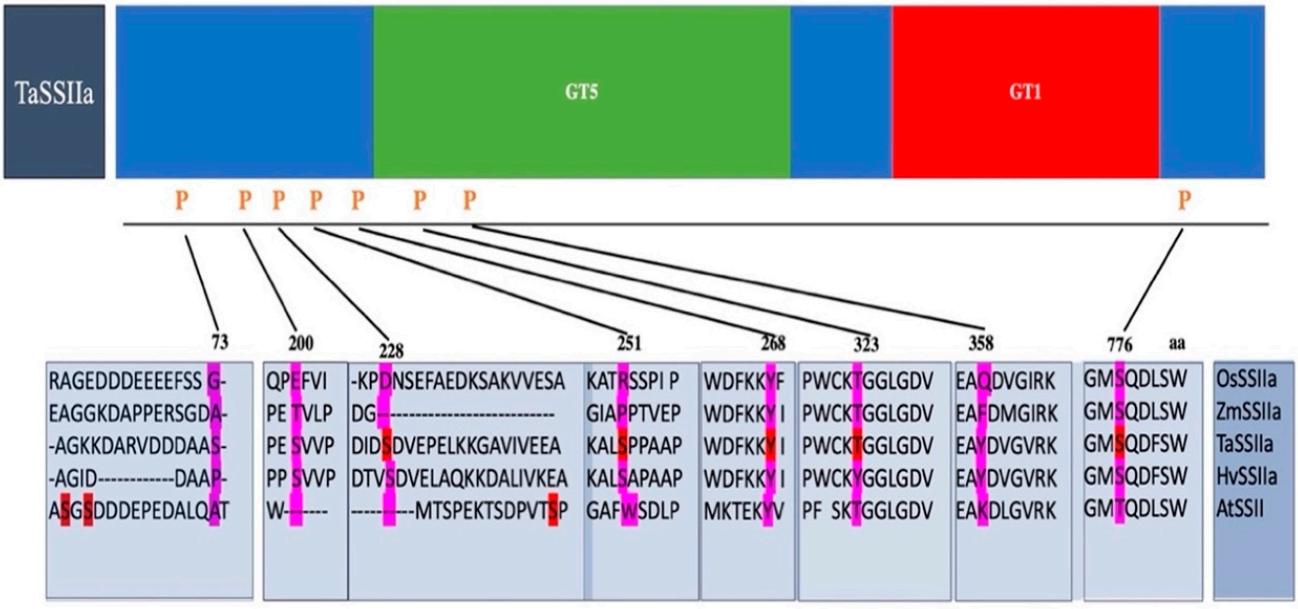

Figure 5. Alignment of SSIIa corresponding regions with different cereal crops and a description of phosphorylation sites of TaSSIIa. Phosphorylation sites are indicated in magenta and red colors were experimentally verified by Chen et al. [37] for wheat SSIIa. GT1: glycosyltransferase family 1; GT5: glycosyltransferase family 5; TaSSIIa: *Triticum aestivum* starch synthase IIa.

4.2. SSs form Heteromeric Protein Complexes in Amyloplasts

Investigating the mechanisms of SS complex formation in plastids is critical for understanding storage starch biosynthesis, which is mainly associated with the enzymes involved in amylopectin synthesis [97]. Different SS classes and SBEs form a trimeric complex and play a role in amylopectin cluster biosynthesis. During the isolation of complex, all three enzymes (SSI, SSII, and BEII) remain catalytically active [35]. It has been suggested that in this trimeric complex, SSII and BEII have a significant level of affinity for amylopectin [22]. It has been depicted that the trimeric protein complex components (SSI, SSIIa, and SBEIIb) become entrapped within the starch granule due to SSIIa glucan binding capacity [64]. Several heteromeric protein complexes are proposed to be involved in amylopectin biosynthesis, which is mainly regulated through protein phosphorylation [98]. An increase in SSI and GBSS levels was observed in *ssIII* endosperms from maize [99] and rice [100]. It can be speculated that alteration in amylopectin is due to overexpression of *SSI*.

5. Conclusions and Future Aspects

Starch synthesis in the endosperm is the basis of yield in almost all-important crops. Starch granule formation in cereal crops needs to be carefully coordinated between SSs, SBEs, and DBEs to form amylopectin clusters. These clusters are the main blocks of water-insoluble polymers. Our present knowledge about the structural and functional understanding of SSs showed that these isoforms are important in starch biosynthesis. This information also provides insight into the post-translational regulation of these enzymes. SSs are essential in starch storage crops, especially to improve starch quality and yield for providing nutrition to people across the globe. From recent research, it is apparent that SSs are subject to protein phosphorylation and the formation of heteromeric protein complexes by association with starch-relevant enzymes. This development helps to further our understanding of this essential biosynthetic pathway. Similarly, new mutation detection methods such as TILLING, TILLING by sequencing or genome-editing can greatly promote to understand the function of soluble starch synthase genes and help to starch improvement. For future studies, there is a need to identify the regulatory enzymes and kinases/phosphatases that are involved in establishing protein-protein interactions, which will provide valuable information for understanding and manipulating starch biosynthesis.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agronomy11101983/s1, Table S1: List of sequences used in this study.

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