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Review

The Structure, Function, and Regulation of Starch Synthesis Enzymes SSIII with Emphasis on Maize

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Abstract: Starch biosynthesis is a complex and highly controlled process that requires coordinated activities among multiple enzymes. Starch synthase III (SSIII) is the largest protein in the starch synthase complex and its function is to lengthen long-chain amylopectin in starch synthesis. It potentially affects the activity of other key enzymes in starch synthesis through protein–protein interactions; therefore, its function and regulation play a predominant role in starch synthesis. In this review, we summarized the main research of SSIII including its biochemical characteristics, structural features, expression atlas, and regulation means. Structural features and expressional analysis indicated that SSIIIa is the main functional protein in maize endosperm rather than SSIIIb-a and SSIIIb-b, even though they are similar in the tertiary structures. The regulation investigation of SSIIIa showed that there are 13 transcription factors that control the transcription of SSIIIa. Interaction network analysis showed that SSIIIa could be involved with ten other key enzymes in starch synthesis. In conclusion, this review considerably extends our understanding of SSIII and provides the theoretical basis for improving starch synthesis by SSIII in maize.

Keywords: maize; starch synthesis; starch synthesis enzymes SSIII; structure; function

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1. Introduction

Maize is one of the major food crops in the world and can be utilized as a resource for food, animal feed, and biofuel [1,2]. Starch is the primary storage form of carbohydrates and the second most abundant biopolymer after cellulose [3]. It is one of the final products of photosynthesis and utilized as an energy source in plants [4,5]. In maize, starch is the main component, accounting for about 75% of its total dry matter content [1]. The production of maize starch dominates the starch industry in the United States and European regions [6].

Starch can be divided into two types: transient starch and storage starch [7]. In photosynthetic tissues, transient starch accumulates in chloroplasts during the daytime. At night, it is hydrolyzed to maltose and glucose (Glc) to support respiration and metabolism [8]. Storage starch is deposited in the specialized compartments (amyloplast) of the non-photosynthetic tissues (endosperm, tuber, and roots) [9]. Furthermore, it can be used not only as a key factor in determining the quality of food or feed products, but also as a cost-effective biodegradable and renewable industrial raw material. It is widely used in industries related to energy, chemicals, medicines, construction, and plastic production [10–12].

In maize endosperm, sucrose is the carbon source for starch synthesis that is transported to the cytoplasm and converted into ADP-glucose (ADPG) by cytosolic ADP-glucose

pyrophosphorylase (AGPase). The ADPG is transported into the amyloplast to form α-glucose-1-phosphate (G-1-P), which is converted into starch through a series of reactions [13]. SSIII forms a complex with AGPase, pyruvate phosphate dikinase (PPDK), starch synthase IIa (SSIIa), starch branching enzyme IIa (SBEIIa), and starch branching enzyme IIb (SBEIIb) [13]. In the complex, SSIII is found as the largest molecular weight protein that resides in a central position and links the phosphate transfer and starch synthesis [13] (Figure 1).

In summary, maize starch dominates overall starch production, and SSIII could significantly affect starch production because of its central position in the enzymes' complex system. The relationship between SSIII and maize starch biosynthesis has not yet been clearly reported. In this paper, we have studied the structure, function, and regulation of SSIII. Moreover, physicochemical properties, expression, protein-level interactions, and regulation at the transcriptional level have also been concluded.

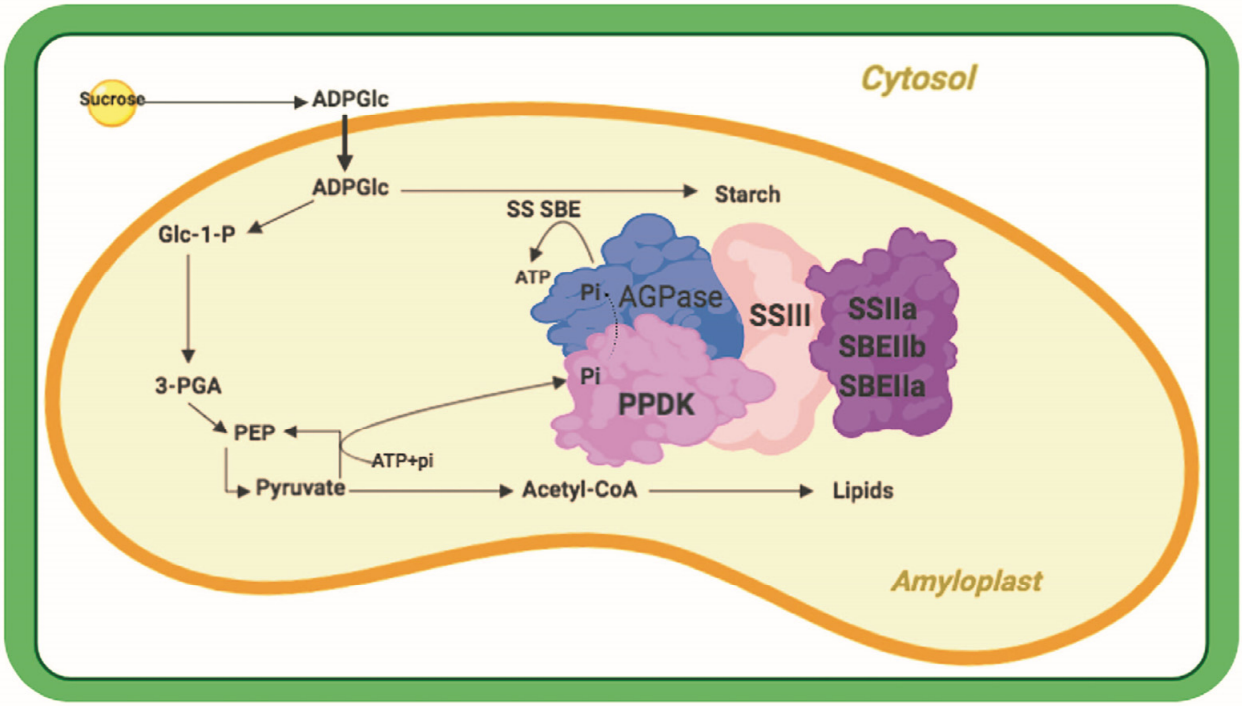

Figure 1. The starch synthesis pathway in maize and the key role of SSIII in the starch synthesis system. Abbreviations; ADPGlc: ADP-glucose, G-1-P: 1-Phosphogluconate, 3-PGA: 3-Phosphoglycerate, and PEP: phosphoenolpyruvate.

2. Function of Maize Starch Synthase III in Starch Synthesis

Starch synthases (SSs) mainly catalyze the formation of alpha-1,4 glycosidic linkages, which are mainly responsible for the elongation of glucosyl chains during starch biosynthesis [14]. In maize, SSs mainly include six isotypes including granule-bound starch synthase (GBSS), SSI, SSIIa, SSIIb, SSIII, and SSIV [15,16], which are divided into two types: GBSSs and soluble starch synthases (SSSs) [17,18]. The main function of the GBSSs is to form amylose, and the SSSs are mainly responsible for the synthesis of branched-chain amylopectin [19,20].

SSIII is found as the second major SS isoform and its activity accounts for 28% of the total activity, which is second only to that of SSI in developing maize and rice endosperm [21,22]. The study of mutants' lines and some in vitro biochemical experiments have inversely explained some functions of SSIII; it is reported that the kernel phenotype of *Dull1* (SSIIIa is encoded by *Dull1* [23]) mutants is opaque and the starch content in the entire kernel of *Dull1* mutants decreased when compared with normal genotypes [24]. Moreover,

previous studies have shown that SSIII may also have the specific function of catalyzing the extension of the long glucan chain in the process of maize starch biosynthesis [25].

The deletion of SSIII has demonstrated that the main function of SSIII is to synthesize amylopectin by polymerization of long chains, which are 30 times greater than the medium chains in *Oryza sativa* and *Hordeum vulgare* [26,27]. The lack of SSIIIa in rice endosperm was reported to affect the structure of amylopectin, amylose content, and the physicochemical properties of starch granules [28].

The glucan chains in amylopectin are divided into A-chains, B-chains, and C-chains, depending upon the point of connection to glucan chains. A-chains are distributed externally with no branches, and B-chains have at least one cluster. The C-chain is a part of the B-chain in a molecule with a free reducing end [29]. Analysis of SSIII mutants of maize identified that fewer long cluster spanning B-chains were present in mutant lines [30]. It has been reported that the deficiency of SSIII led to an increased number of short chains, whereas the content of long chains decreased in amylopectin [31]. In addition, there are indications that the *Dull1* mutation resulted in the larger cluster in branches in amylopectin, with more singly branched building blocks [32] (Figure 2).

Therefore, it can be suggested that the role of SSIII is to form highly ordered amylopectin. The mutants lacking in SSIII would affect the synthesis of amylopectin, reduce the synthetic amount of starch, change the structure, affect the grain weight, and increase the ratio of amylose in starch [33,34].

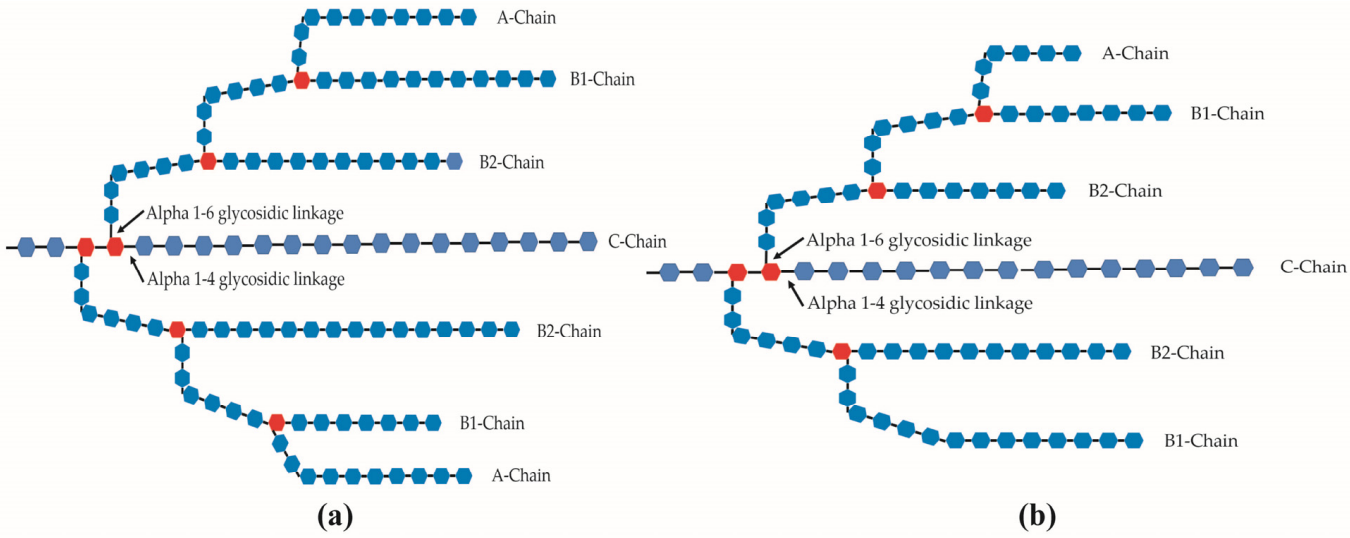

Figure 2. Structural features of maize amylopectin. (**a**) Pattern diagram of maize amylopectin in wild type maize. The hexagons indicate glucose molecules and the short horizontal lines indicate glycosidic bonds. (**b**) Pattern diagram of maize amylopectin in the *Dull1* mutants. The hexagons indicate glucose molecules and the short horizontal lines indicate glycosidic bonds. The *Dull1* mutations lead to fewer long clusters on B-chains, an increasing number of short chains, and a larger cluster in branches in amylopectin with more singly branched building blocks.

3. Physicochemical Properties of Maize Starch Synthase III

It has been reported that maize SSIII consists of two isozymes, SSIIIa and SSIIIb, which are most likely ascended from the ancient duplicate sets in the Gramineae. SSIIIb further consists of SSIIIb-a and SSIIIb-b isoforms, which probably arose from the second duplication event in maize [35]. The cDNA of *Dull1* is 5025 base pairs (bp) long and comprises 15 introns and 16 exons. The length of the first three exons is found to be variable, while the other 13–16 exons are the same in length. SSIIIa encodes 1675 amino acids (aa) with a predicted molecular weight of 188 kDa [36]. The sequence of maize SSIIIa has divided into four different regions: a specific transport peptide region, a *Dull1* specific N-terminal region, a central region (homologous to other class III Starch Synthases), and a C-terminal

region containing a catalytic domain [23]. In addition, a conserved carbohydrate-binding module was identified in the N-terminal region, which plays an important role in substrate binding [7].

We have analyzed the domains and functional sites of maize SSIII protein utilizing the Conserved Domains Database (CDD) and SMART database. It is found that the SSIII contains a coil coiled (CC) domain at the N-terminal, which could participate in protein–protein interaction [37]. In addition, a comparative study has indicated that different isoforms of starch synthases in *Zea Mays*, *Oryza sativa*, and *Arabidopsis thaliana* consists of a basic domain that belongs to Glycosyltransferase family 5 (GT5) and Glycosyltransferase family 1 (GT1). However, the carbohydrate-binding module of family 25 (CBM25) is unique in the SSIII, which may be related to the fact that SSIII is mainly responsible for the synthesis of long glucan chains [38]. CBM has been described as a contiguous amino acid sequence within a carbohydrate-active enzyme, with a discrete fold that possesses carbohydrate-binding activity [39]. The importance of CBM has been shown in *Arabidopsis thaliana*, as the mutant lack in SSIII displayed higher starch accumulation in leaves during the daytime [40].

In maize, significant variations have been observed in the distribution of domains between GBSSs and SSs. Similarly, motif analysis has also indicated the differences among motifs in the domains of GBSSs and SSs. The distribution of conserved motifs in the GT5 and GT1 domains of SSIII differs a little with other SSs but more with GBSSs. Interestingly, in comparison to other isoforms, a unique motif (motif 23) in the GT5 domain has also been identified in SSIII (Figure 3). Future studies are needed to demonstrate the exact function of motifs in SSs, which are currently predicted to be involved in the synthesis of the tertiary structure of starch synthases, binding to ADP, and maintaining the stability of the structural domain [29].

Tertiary structures of SSIIIa, SSIIIb-a, and SSIIIb-b being performed by the online software AlphaFold [41,42], and the CBM25, GT5, and GT1 structural domains were highlighted by UCSF Chimera. The model confidence of the structural domains area is very high (pLDDT > 90), despite the large differences in the lengths of the aa sequence between SSIIIa, SSIIIb-a, and SSIIIb-b. The spatial distribution of the structural domains showed consistency. In the CBM structural domains, β-pleated sheets are found to be dominant, while in the GT structural domain, more α-helixes are recognized (Figure 4). Studies are required to demonstrate the relationship between the distribution of the unique tertiary structure and the function of the structural domain. The exact relationship between the tertiary structure of the peptide chain and the structural domain of SSs needs to be addressed.

Fewer studies have demonstrated the chemical properties of SSIII. It has been shown that the maize SSIII demonstrates its maximum activity rate at 37 °C, whereas it maintains 60% of its maximum activity at 23 °C and the activity decreases by more than 50% at 42 °C [36]. A similar pattern of activity was observed using the recombinant maize SSIII [43]. Exploring the chemical conditions under which SSIII functions optimally can help in genetic breeding research to identify the key position of SSIII in maize starch synthesis.

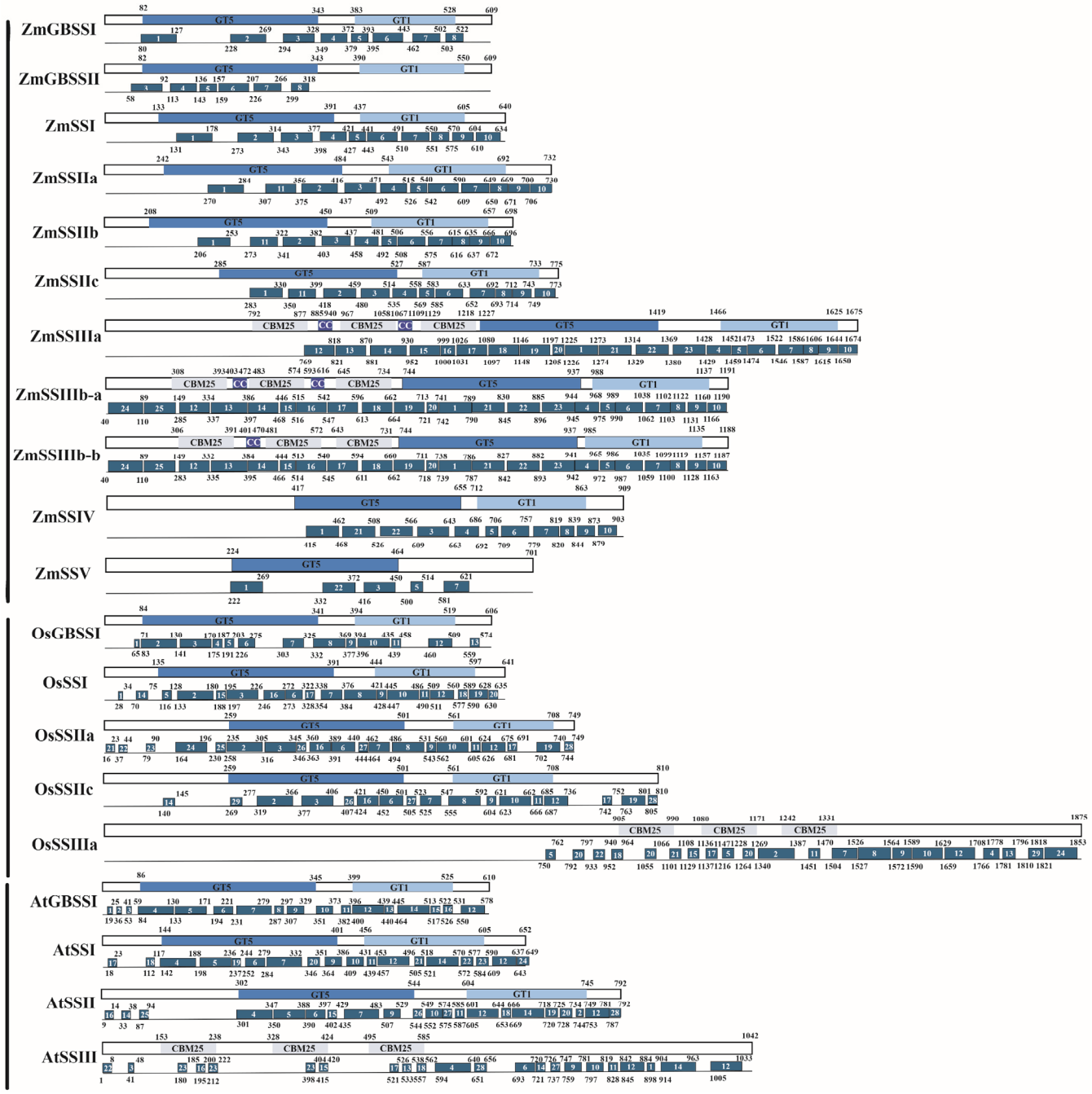

Figure 3. Composition and distribution of domain structures and conserved motifs of SS proteins. *Zea Mays* (Zm), *Oryza sativa* (Os), and *Arabidopsis thaliana* (At) are shown as an example. The N-terminus is on the left side of the figure and the C-terminus is on the right side. The three functional structure domains GT, CBM, and CC are marked in black font, and the predicted conserved motifs are marked in white font. The detailed gene list is in Supplementary Table S1.

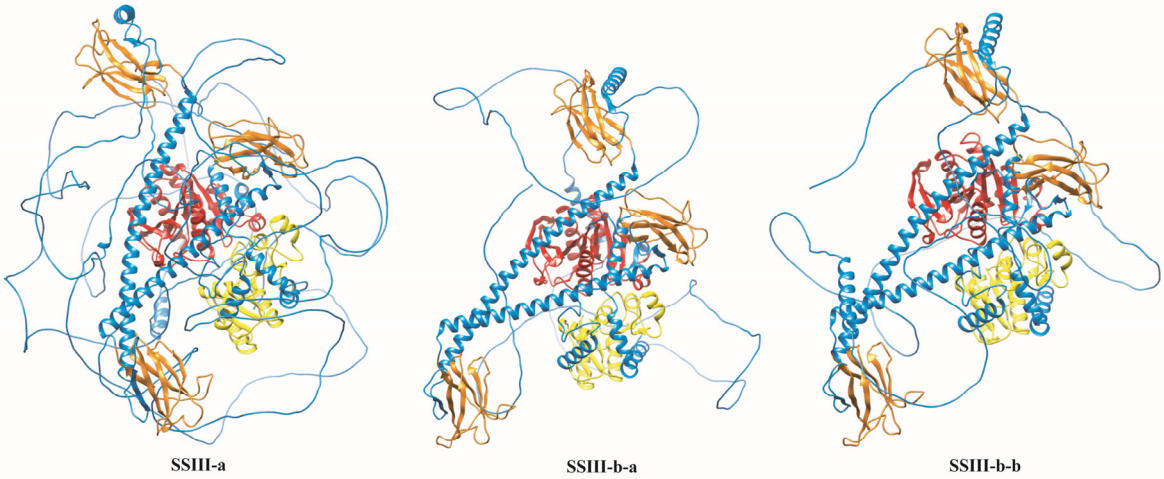

Figure 4. Tertiary structure prediction of SSIII genes, CBM25, GT5, and GT1 structural domains are highlighted in orange, red, and yellow colors, respectively. It can be seen that the spatial distribution of structural domains showed consistency. Consistent spatial distribution laterally reflects that the structural domains could play a central role in SSs function.

4. Temporal and Spatial Expression Analysis of Starch Synthase Gene SSIII in Maize

Dynamic expression patterns of SSIII have been explored by RNA-sequence data analysis [44]. It has been identified that SSIIIa begins to express in the early stage and could be detected in the endosperm six days after pollination (DAP). The expression reaches its maximum at 16 DAP, and then shows a downward trend until 28 DAP. The expression at 30 DAP was found to be approximately equivalent to that at 3 DAP. Meanwhile, the expression level of SSIIIb-a and SSIIIb-b in the endosperm was found significantly lower than SSIIIa. Interestingly, the expression pattern of the mentioned isoforms in embryos was found to be similar. In the phase of 10 DAP to 38 DAP, the expression of SSIIIa and SSIIIb-a decreases gradually, while the expression of SSIIIb-b continues to rise (Figure 5).

SSIIIa is expressed at higher levels from approximately 10 DAP to 30 DAP than during the early and late phases of whole seed development. This phenomenon has been predicted to be related to the process of seed and cell development of the maize. The phase of 0–10 DAP is the key period of cell proliferation and differentiation, while 10–30 DAP is the period of the rapid growth and maturation of the embryo and the endosperm [44].

In terms of spatial differences in SSIII expression, SSIIIa is mainly expressed in the endosperm while the SSIIIb-a is mainly expressed in leaves, and weakly so in roots and endosperm (Figure 5). SSIIIb-b is highly expressed in cob and silk, but moderately expressed in leaves; its expression is according to this leaf > vein > root pattern [29,36]. A previous study has determined the expression patterns for SSIIIb-a and SSIIIb-b by semi-quantitative RT-PCR analysis and the result showed that SSIIIb-a is mainly expressed in leaves but weakly expressed in the roots and endosperm. Similarly, SSIIIb-b is highly expressed in roots and early developing endosperm, but moderately expressed in leaves [35].

Based on the high expression of SSIIIa in the seeds as well as in the endosperm, it can be speculated that maize starch synthesis could be regulated by SSIIIa via direct function or interaction with several starch synthetic enzymes, which may be related to the presence of longer N-terminal and shorter C-terminal amino acid sequences of SSIIIa (Figure 4).

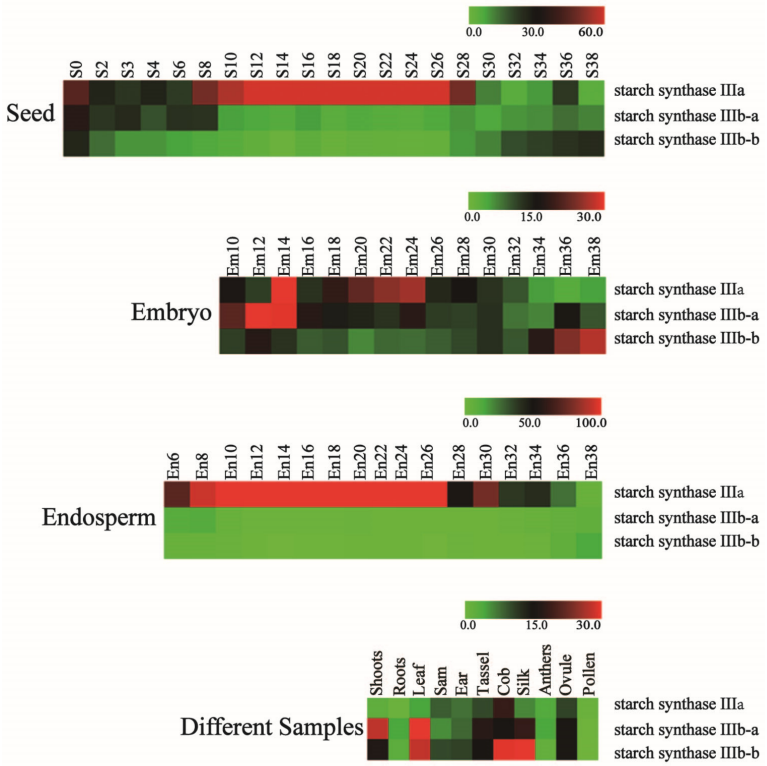

Figure 5. The expression level of SSIII genes in different tissues. In maize seed, the expression levels of SSIIIb-a and SSIIIb-b maintain a low level, while there was a high-level expression phase of SSIIIa from day 8 to 28, showing an overall trend of first increasing and then decreasing. In the embryo, the expression levels of SSIIIa, SSIIIb-a, and SSIIIb-b are found to be higher than those in the seeds. In the endosperm, the expression of SSIIIa is found to be dominant, with the highest expression occurring from 12 to 26 DAP; the expression showed an overall trend of increasing and then decreasing from 6 to 36 DAP. In addition, by analyzing the expression pattern in other, different tissues of maize, it is found that the expression of SSIIIa is low in all other samples except in cob; SSIIIb-a expression is high in shoots and leaves; SSIIIb-b expression is high in cob and silk [44]. The data used for graphing are listed in Supplementary Table S2.

5. Protein-Protein Interactions and SSIII

5.1. Regulation of SSIII on Other Key Starch Synthases

In addition to its enzymatic function, SSIII could act as a "regulator" of starch biosynthesis as its activity and expression have been found related to other SSs [45]. Recently, it has been confirmed that SSIII can affect the activities of other key enzymes in starch biosynthesis through protein–protein interactions [46]. A large number of pairwise interactions by yeast two-hybrid detection, immobilized recombinant ligand affinity purification, and immunoprecipitation involving SSs and SBEs have been demonstrated in maize. Positive interaction between SBEIIa and SSIIIa$^{1-367}$ (fragment of the full-length SSIIIa) in yeast (*Saccharomyces cerevisiae*) nuclei has already been identified [47]. Meanwhile, another two separate regions of SSIIIa (residues 1 to 367 and residues 760 to 1438) are found to interact with full-length SSI in the in vivo interaction test [47].

The study of the maize *Dull1* mutant has shown that the activity of SSII was greatly lower than that of the wild type, suggesting the regulatory role of SSIII [35]. Likewise, the activities of SBEIIa and SSIIa were found to be lower in the extracts from maize kernels that were homozygous for the recessive *Dull1* allele [48]. Recently, it has been reported that the SSIII promotes the expression levels of SSI and GBSSI in the maize endosperm [6].

The regulatory role of SSIII has also been demonstrated in *Arabidopsis thaliana;* the activity of other isoforms of SSs were enhanced under overexpression of *Dull1* [49]. SSIII is found closely related to SSIV and it can be speculated that the role of SSIV in granule

seedlings can be partly replaced by SSIII, as the elimination of SSIV does not affect the synthesis of starch [49]. It has been identified that the simultaneous elimination of both proteins prevents *Arabidopsis thaliana* from synthesizing starch [49].

In this study, a protein–protein interaction analysis was performed for SSIIIa. The results of the analysis showed that interactions exist between SSIIIa and 10 other proteins. Interaction networks have suggested that SSIIIa could be involved in the process of starch biosynthesis and cellular glucan metabolism. In terms of molecular functions, SSIIIa has glycogen (starch) synthase activity, starch-binding activity, and transferase activity for transferring hexose moieties. Several interaction partners of SSIIIa are speculated from KEGG's PATHWAY database, and these interacting partners suggest that SSIIIa could be involved in starch metabolism, sucrose metabolism, and the biosynthesis of secondary metabolites (Figure 6).

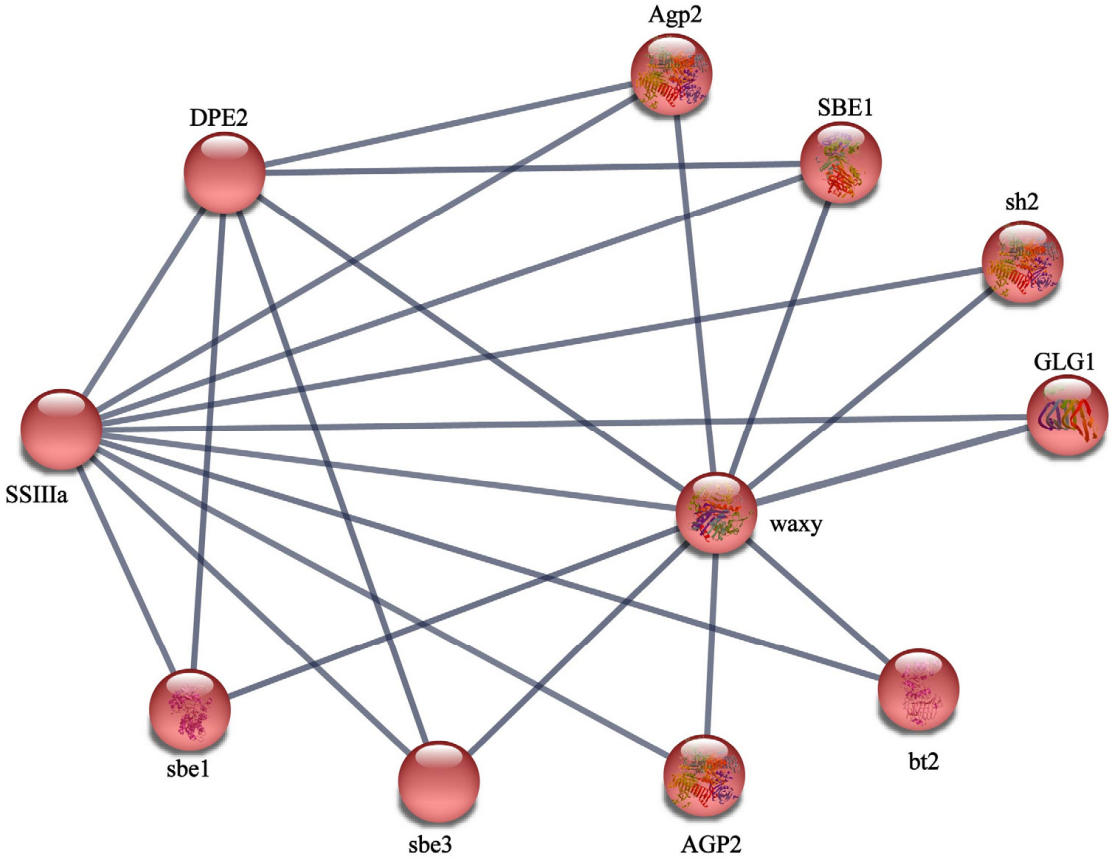

Figure 6. The network of interactions is proven by curated databases or experiments between 10 key enzymes and SSIIIa in starch synthesis; maize is shown as an example. The interaction network suggests that SSIIIa can be involved in the process of starch biosynthesis and cellular glucan metabolism. The abbreviated gene name corresponds to its full length. SSIIIa: starch synthase IIIa; DPE2: 4-alpha-glucanotransferase DPE2; Agp2: ADP-glucose pyrophosphorylase 2; SBE1: amylose extender 1; sh2: shrunken 2; GLG1: ADP glucose pyrophosphorylase small subunit leaf 1; waxy: Granule-bound starch synthase 1; bt2: Glucose-1-phosphate adenylyltransferase; AGP2: ADP-glucose pyrophosphorylase; sbe3: starch branching enzyme 3; sbe1: starch branching enzyme 1. Empty nodes: proteins of unknown 3D structure, filled nodes: known or predicted 3D structures. Genes and protein sequences are listed in Supplementary Table S3.

5.2. Enzyme Complex Involving SSIII

Physiological and biochemical studies have shown that each isoform of SSIII exists in the form of an enzyme complex and starch synthesis-related enzymes constituting the complex that affects the activity of SSIII. In starch synthesis, key enzymes are assembled

into well-coordinated complexes in which the physical association of SSIII with other enzymes plays a key role in regulating the fate of the complex [50].

In maize, SSIIIa was discovered from a high molecular complex of approximately 600 kDa, which was stable at high salt concentrations through gel permeation chromatography [47]. Recently, it has been reported that SSIII, SSIIa, SBEIIa, and SBEIIb are most likely to exist together in a 670kDa complex in maize. The elimination of any of these enzymes prevents others from assembling into a complex [13]. Furthermore, a high molecular weight multiple enzyme complexes composed of SSIII, SSIIa, SBEIIa, and SBEIIb is also found in maize, which is further assembled into a 670 kDa complex by interacting with PPDK and sucrose synthase isoform SH1 (SUS-SH1). These protein complexes may play a role in the overall regulation of carbon distribution between the metabolic pathways of developing seeds [13].

SSIII was also found to co-exist in complex with other key enzymes related to starch biosynthesis in several model crops. A protein complex of more than 700 kDa in size has been identified through gel permeation chromatography and immunoprecipitation in the endosperm of *Oryza sativa* and found to be made of SSIIa, SSIIIa, SSIVb, SBEI, SBEIIb, and Pullulanase (PUL), while SSI, SSIIa, SBEIIb, Isoamylase 1 (ISA1), PUL, and plastidial starch phosphorylase (PHO1) also co-eluted the 200–400 kDa complex [51].

5.3. Protein-Protein Interactions Based on Phosphorylation

Reversible phosphorylation of protein is a key post-translational modification of proteins and acts as a key regulatory mechanism for many proteins' functions, which are related to many biological processes [52]. At present, many proteins related to starch synthesis and regulation are found phosphorylated during the development of maize grains [53–56]. Protein phosphorylation regulates the component interaction and plays a pivotal role in the formation of enzyme complexes [57]. Previously, multiple phosphorylation sites have been identified in the maize SBEIIb and were found to affect the formation of the complex [58]. Furthermore, studies have found that SSII can form more stable complexes with SSIIa, SBEIIa, and SBEIIb when one of the residues in SSII is modified via phosphorylation [58]. It is found that the treatment of maize endosperm extract with alkaline phosphatase prior to immunoprecipitation seriously reduces the amount of SSIIa, SBEIIa, SBEIIb, and PPDK, which are co-precipitated with SSIII. In contrast, the addition of protein phosphatase inhibitor NaF to the extract increases the level of mentioned proteins in the immunoprecipitation [59].

It is a matter of fact that changes in protein activity usually require the interaction of phosphorylated proteins with adapter "14-3-3" proteins [60]. Immunoprecipitation and Western blotting experiments have ascertained that the SSIII protein of maize could also bind with the 14-3-3 protein [61]. The removal of phosphate groups reduces or eliminates the co-immunoprecipitation signal. Moreover, several novel complexes which comprise large and small subunits of AGPase, SBEIIa, SBEIIb, SSIIa, SSIII, and PPDK with phosphorylase (PHO1) in a phosphorylation manner in maize, wheat, barley, and rice have been previously described [62].

Therefore, it can be suggested that the phosphorylation of starch synthase and the interaction between enzymes could play an important role in regulating starch synthesis and determining the modification of SSIII via phosphorylation. Additionally, it could be interesting to know the key residues of the phosphorylation.

6. Regulation of Maize SSIII on Transcription Factors Level

6.1. Isolation, Characterization, and the Functional Determination of Dull1 Promoter

Recently, the promoter of *Dull1* (*PDull1*) flanking on the upstream 5′ region of *Dull1* in maize has been isolated and characterized [63]. Some putative *cis*-acting regulatory elements (Skn-1_motif, GCN4_motif, and AACA motif) that regulate the expression of SSIII in the endosperm have been identified in *PDull1* [63]. Several motifs (TATA-box, CAAT-box, light-responsive elements, ABRE site, MBS, LTRE, and GT1GMSCAM4) that

are known to regulate organ and tissue-specific gene expression have also been identified in *PDull1* [63].

In addition, it has been reported that the upstream 5′ flanking region of the *Dull1* contains the important regulatory sequences (−343 to 1 bp), which control the expression of SSIII in the endosperm. *PDull1* has been found to regulate the β-glucuronidase (GUS) activity in the endosperm of *Oryza sativa*, suggesting that *PDull1* could act as a potential tool to increase crop yield [63].

6.2. Regulation of SSIII by Transcription Factors

Starch synthesis and starch-related proteins have been intensively studied as these two components limit the value of grains in cereal crops; the transcription factors that regulate starch synthase remain largely unknown. At present, a limited set of transcription factors have been identified in plants and mainly include AP2/EREBP, bZIP, NAC, MYB, ARF, and WRKY, which are involved directly (physical binding) or indirectly (functional interaction attributed to other binding partners) in the regulation of starch biosynthesis through modulating the starch biosynthetic enzymes. The research on the direct regulation of starch biosynthesis via transcription factors in cereals has received relatively less attention.

In rice, rice starch regulator 1 (RSR1)–which is an AP2/EREBP family transcription factor–has been found to regulate the expression of starch synthesis genes [64]. WRKY transcription factor (SYSIBA2) has been reported to regulate the expression of starch synthesis genes in barley [65]. There are only a few transcription factors that have been reported to regulate the expression of starch synthesis-related genes in maize so far. Recently, it has been identified that ZmNAC34–which is specifically expressed in maize endosperm–can negatively regulate starch synthesis [66]. Moreover, ZmaNAC36 has been recently characterized and found to participate in the regulation of starch synthesis; expressional analysis indicated its extremely low expression in roots, stems, embryos, and flowers when compared to its expression in maize endosperms [67]. In addition, it has been suggested that ZmaNAC36 can be involved in the co-expression of many starch synthetic genes such as SSs, GBSSs, and SBEs [67]. It has been reported that ZmNAC128 and ZmNAC130 regulate the transcription of bt2 and reduce the protein level, which acts as a rate-limiting step in the starch synthesis of maize endosperm [68]. Meanwhile, ZmNAC128 and ZmNAC130 have been found to affect the expression of zein genes and reduce the accumulation of zeins and non-zeins proteins [68]. Several transcription factors that influence the SSIII directly or indirectly have been identified are listed in Table 1.

Table 1. Information, expression characteristics, and regulation of transcription factors regulating SSIII.

Transcription Factors' Name	Amino Acid	Characteristics of Expression	Interaction with SSIII	Reference
ZmEREB156	233 aa	Expresses in the roots, stems, leaves, and endosperm; the expression is higher in the endosperm and leaves. Expression is regulated by sucrose or abscisic acid (ABA) and may be synergistically regulated by sucrose and ABA.	Binds directly to the *PDull1* and promotes the expression of SSIII.	[69,70]
ZmEREB192	329 aa	Expresses highly in different periods of post-pollinated maize endosperm. Expression is upregulated by sucrose, downregulated by ABA, and significantly upregulated by sucrose and ABA.	Binds to the *PDull1* weakly and suppresses the activity of *PDull1*.	[71]

Table 1. *Cont.*

Transcription Factors' Name	Amino Acid	Characteristics of Expression	Interaction with SSIII	Reference
ZmEREB25	292 aa	Expresses highly in different periods of post-pollinated maize endosperm. Expression is upregulated by sucrose and ABA, and significantly upregulated by sucrose and ABA.	Binds directly to the *PDull1* and promotes the activity of *PDull1*.	[71]
ZmEREB26	428 aa	Expresses highly in different periods of post-pollinated maize endosperm.	Promotes the activity of *PDull1*.	[72]
ZmEREB94	283 aa	Expresses in the stem, seed, endosperm, and embryo, and the expression is higher in the embryo and endosperm. Expression is upregulated by sucrose and ABA.	Correlate with the expression of SSIII	[73]
ZmbZIP91	563 aa	Expresses highly in maize endosperm, strongly associated with the expression of SS genes.	Binds directly to the ACTCAT element of *PDull1*.	[74]
ZmbZIP22	183 aa	Expresses at a relatively high level in the endosperm, and the level of expression increases gradually after pollination.	Binds directly to the ACGT element of *PDull1*.	[75]
Opaque2	405 aa	-	Binds directly to the O2 box (ACGT element) of *PDull1* and exerted strong activation on its transcription	[76]
ZmWRKY82.2	613 aa	Expresses highly in the embryo and different periods of post-pollinated maize endosperm. Expression is upregulated by sucrose, downregulated by ABA, and significantly upregulated by sucrose and ABA.	Binds directly to the *PDull1*.	[77]
ZmPLATZ2	309 aa	The expression is highest at 12 DAP and decreases rapidly post 12 DAP. Expression is upregulated by glucose and downregulated by sucrose and ABA.	Promotes the activity of *PDull1*.	[78]
ZmNAC126	322 aa	Expresses highly in the endosperm.	Promotes the activity of *PDull1*.	[79]
ZmMYB115	517 aa	-	Significantly suppress the activity of *PDull1*.	[80]
ZmGRAS20	508 aa	Expresses highly in the endosperm.	Promotes the activity of *PDull1*.	[72]

6.2.1. Regulation of Transcription Factors and Signal Molecules

Transcriptional regulation plays a key role in many regulatory pathways of starch biosynthesis and transcriptional regulation, often coupled with related signal molecules such as sucrose and hormones. It is reported that higher gibberellic acid (GA) content can reduce the activity of starch biosynthetic enzymes during rice grain filling [81,82]. Similar regulation exists in maize SSs, and most of the biochemical pathways are associated with ABA, sucrose, and glucose. Sucrose and ABA can regulate the expression of genes related to maize starch synthesis, including *Dull1*, and it has been found that gene expression treated with sucrose and ABA was higher than that treated with sucrose alone [83]. In maize, the activity of *PzsS3a*–which is a motif in the promoter of SSIII–has been reported to be induced

by ABA treatment [84]. The expression of the ZmEREB94 gene was found to correlate with SSIIIa, and its RT-qPCR analysis showed that the expression of ZmEREB94 noticeably decreased after 0.5 h of sucrose treatment and was only slightly affected by the ABA and glucose treatment [73]. The transcription factor ZmEREB156, which can bind to the promoter of SSIII and positively regulate its expression, has also been reported to regulate the expression of genes related to starch synthesis in maize endosperm by responding to sucrose or ABA signals [69]. It has further been reported that ZmWRKY82.2 is also co-induced by sucrose and ABA. However, the specific mechanism of this co-regulation has not been analyzed [76]. The expression of ZmPLATZ2 is found to be strongly upregulated by glucose and temporarily downregulated by sucrose; critical downregulation has been found by adding ABA [78].

6.2.2. Regulation of Transcription Factors by Micro RNA

Micro RNA (miRNA), which is a class of short non-encoding RNA, can regulate gene expression after the transcription through sequence complementarity. Recently, it has been reported that miRNAs play essential roles in regulation and that networks mediated by miRNA could modulate starch biosynthesis in maize endosperm [85]. At present, only a few miRNAs have been reported to be involved in the regulation of starch biosynthesis.

Studies have shown that the overexpression of miRNA *Crongrass1,* which belong to the miR156 subgroup, can increase the starch content in plants [86]. Regarding the regulatory pathway of miRNA on starch synthase, recent studies have shown that miRNAs form a regulatory pathway (miRNA-transcription factor-SSs) with transcription factors and SSs [85]. The transient expression of two miRNAs—*miR169a* and *miR169o*–and transcription factors–nuclear transcription factor Y subunit A1 (NF-YA1) and C2C2-GATA-transcription factor 9 (GATA9)–in maize endosperm showed that miR169a could negatively regulate the expression of NFYA1 and miR169o could positively regulate the expression of GATA9. Meanwhile, SSIIIa was found to be significantly up-regulated via the overexpression of NF-YA1 and GATA9 transcription factors in endosperm, suggesting NFYA1 and GATA9 could enhance the promoter activity of SSIIIa [85]. In addition, bioinformatics predictions have shown that SSIIIa could be the potential target for newly discovered miRNA *PC-3P-336668_6* and *PC-3p-169926_13* [85]. Similar regulatory pathway exists between the transcription factor ZmMYB115 and miRNA *Zma-miR159k-3p,* which could negatively regulate the expression of ZmMYB115 in maize endosperm [80].

7. Concluding Remarks and Future Perspectives

The biosynthesis of starch is a highly complex metabolic process that requires the synergistic action of multiple enzymes. Studies targeting its synthetic pathways have been a long-standing research hot-spot, while there are still ongoing controversies and corrections [87]. Existing studies have shown that SSIII acts as the core and regulatory enzyme of maize starch synthase, with several important functions including synthesizing highly ordered branched starch, influencing the structure of branched starch, and catalyzing the formation of longer sugar chains.

Regarding its physicochemical properties, SSIII was found to consist of a specific transport peptide region. The structure is characterized by a *Dull1*-specific N-terminal region, a central region (homologous to other class III starch synthesis), and a C-terminal region containing a catalytic structural domain for a total of four functional regions. Several other structural domains including CC, GT5, GT1, and CBM25 have been identified in SSII. However, no direct studies have been conducted to the date to demonstrate the functional link of the structural domains with the function of SSIII in starch synthesis. Despite the variations in the sequences, the predicted tertiary structures showed consistency in the spatial distribution of the structural domains in each of the mentioned isoforms, which have not yet been characterized. In terms of its optimal working conditions, only a preliminary study that described the effect of temperature on SSIII is available. Considering the central

position of SSIII in starch synthesis, further investigation of the optimal physiological conditions of SSIII could be significant for adjusting cultivation practices in maize.

The expression of SSIII is found at a maximum during 10–30 DAP, and expression of SSIIIa is dominant in the endosperm. The expression of the starch biosynthetic enzyme significantly affects the starch content of the seeds in these stages, suggesting SSIII could also affect starch yield. The expression results can be utilized in the breeding work of transgenic maize varieties.

In relation to the regulation of metabolic pathways, several studies have shown that SSIII can be a "regulator" of other key enzymes for starch synthesis, including SBEs, other SSs, and GBSSs, which act directly or indirectly on participating enzymes. Several reports suggested that SSIII is involved in both starch biosynthesis and cellular glucan metabolism; it is reported to perform several biological activities including glycogen (starch) synthesis activity, starch-binding activity, and transferase activity for transferring hexose molecules. SSIII in multiple enzyme complexes with SBEs, SSs, ISA, PUL, PHO, and other key enzymes for starch synthesis has been reported in maize and rice crops. However, the pathways of enzyme complex formation and the specific catalytic functions of each part of the enzyme complexes have not yet been characterized. Furthermore, several enzymes in a complex were reported to be modified via phosphorylation, and the exact fate of phosphorylation needed further clarification. Phosphorylation of starch synthases is thought to be a common phenomenon [53]; it can be speculated that SSIII can also modify via phosphorylation since no direct evidence for the phosphorylation has been reported so far, and further studies are needed.

Several key elements regulating starch synthesis have been identified in *PDull1*, however experimental verifications are missing. Further studies are needed to demonstrate the functional importance of the identified elements. Transgenic experiments on *PDull1* have shown that *PDull1* acts as a potential tool to improve crop yield. Transcription factors have been identified to regulate the starch synthesis process in plants. It can be speculated that transcription factors regulating SSIII could be influenced by several signaling molecules, including sucrose, ABA, miRNAs, and a set of several other interacting molecules. Considering the important regulatory role of transcription factors, we need detection and identification of transcription factors, signaling molecules, and miRNAs in future studies, which could help to determine the underlying pathways of signaling molecules and miRNAs for the regulation of SSIII function by transcription factors.

8. Methods

8.1. Data Collection

In this study, 41 gene sequences and protein sequences of three plants were collected. All SS and GBSS genes were downloaded from Maize GDB (https://www.maizegdb.org/, accessed on 1 February 2022) and NCBI (https://www.ncbi.nlm.nih.gov/, accessed on 1 February 2022). All amino acid sequences (domain signature GT1, GT5, and CBM25 for SS) of Maize (*Zea mays* L.), Rice (*Oryza sativa* L.), and Arabidopsis (*Arabidopsis thaliana* L. Heynh) SS genes were downloaded from Uniprot (https://www.uniprot.org/, https://www.ncbi.nlm.nih.gov/, accessed on 1 February 2022).

8.2. Sequence Retrieval and Motif Analysis

To obtain domain signatures (GT1, GT5, and CBM25 for SS, GBSS), PFAM (http://pfam.xfam.org/search, accessed on 1 February 2022), CDD (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.-cgi, accessed on 1 February 2022), and SMART (http://smart.embl-heidelberg.de/, accessed on 1 February 2022) databases were used. All amino acid sequences of maize SS and GBSS genes were analyzed with MEME (v5.4.1) to discover novel conserved patterns with default settings; all E-values are less than 10e-10. (https://meme-suite.org/meme/index.html, accessed on 1 February 2022).

8.3. Tertiary Structure Prediction of SSIII Genes

The tertiary structure of the SSIII gene was predicted by the online software AlphaFold (https://www.alphafold.ebi.ac.uk/, accessed on 1 February 2022); the CBM25, GT5, and GT1 structural domains were highlighted by UCSF Chimera (1.16) with default settings.

8.4. Heat Map Expression Trend Analysis

The MeV (v4.9.0) software (https://sourceforge.net/projects/mev-tm4/files/mev-tm4/, accessed on 1 February 2022) was used for heatmap expression trend analysis. The data were log2-normalized and kept to two decimal places.

8.5. Protein-Protein Interaction Analysis

The online software STRING functional protein association network (https://cn.string-db.org/, accessed on 1 February 2022) was used to analyze protein–protein interactions, and Cytoscape software (http://www.cytoscape.org/, accessed on 1 February 2022) was used for visualization analysis.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy12061359/s1. Table S1: Genes of SSs in *Zea Mays*, *Oryza sativa*, and *Arabidopsis thaliana* used in Figure 3. Table S2: The data used for graphing Figure 5. Table S3: Genes of interaction proteins used in Figure 6.

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