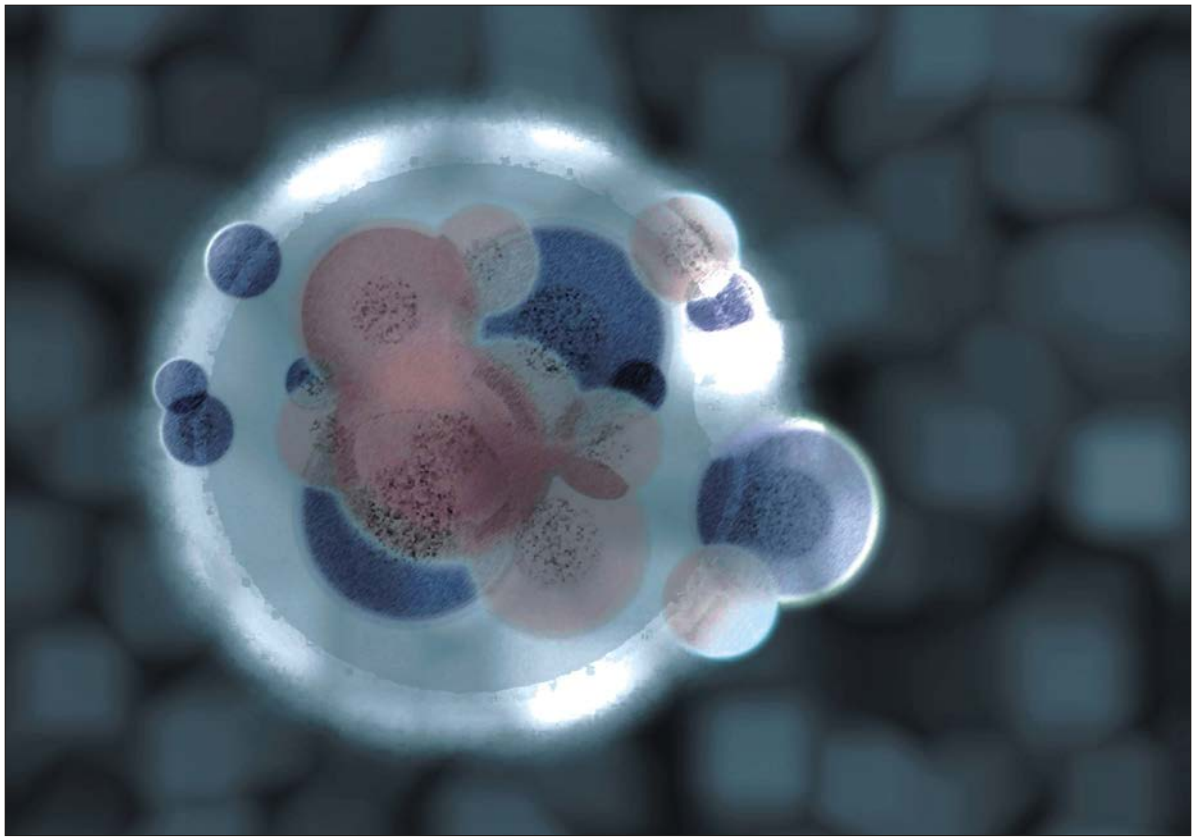




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Molecular Characterization of SSI Gene in *Triticum L.* and *Aegilops tauschii*

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Abstract: In order to knowing the variation of starch synthase I (SSI) in *Triticum L.* and *Aegilops* and exploiting new gene resources for the improving of starch quality in common wheat, using PCR cloning, eight sequences of SSI genes were obtained from AS60 (DD, *Aegilops tauschii*), AS2255 (AABB, *Triticum turgidum*), SHW-L1 (AABBDD, synthetic hexaploid wheat) and Chuanmai 32 (AABBDD, *Triticum aestivum*), respectively. These sequences of SSI gene showed the higher conservative characterization between different materials. A total of 10 variable nucleotide bases and two substitutions of amino acid residues (Ser/Asn and Ala/Val) were observed in the cloning sequences. Alignment of all sequences, including SSI gene from *T. aestivum*, *Hordeum vulgare*, *Brachypodium distachyon*, *Zea mays* and *Oryza sativa* suggested that the sequences from *Ae. tauschii*, *T. turgidum* and synthetic hexaploid wheat, were more similar to SSI gene from common wheat and barley and far distance with sequences from *Brachypodium distachyon*, *Zea mays* and *Oryza sativa*. Using neighbor-joining method, phylogenetic tree including a total of 28 sequences could be clustered into four groups. Most of sequences of SSI from different species were clustered together and SSI gene showed the clear difference with other starch synthases, including Granule-bound Starch Synthase (GBSS), SSII, SSIII and SSIV. These results suggested that there was variation from SSI gene in *Triticum L.* and *Aegilops*. It necessary to developing molecular markers for exploit genetic resources and the improving of wheat starch quality.

Key words: *Aegilops tauschii*, starch synthase, *Triticum L.*, phylogenetic tree

INTRODUCTION

Starch synthase (SS) plays an important role in the series of synthesis of starch of wheat. SS extend α -1, 4 glucan chains by catalyzing the transfer of the glucosyl moiety of ADP-Glc to the reducing end of a pre-existing α -1,4 glucan primer to synthesize the insoluble glucan polymers amylose and amylopectin. Four soluble starch synthase gene: SSI (Baba *et al.*, 1993; Knight *et al.*, 1998), SSII (Dry *et al.*, 1992; Edwards *et al.*, 1995; Harn *et al.*, 1998), SSIII (Abel *et al.*, 1996; Marshall *et al.*, 1996; Gao *et al.*, 1998) and SSIV are each thought to be primarily involved in amylopectin synthesis and also play the roles in amylose biosynthesis. Each type of starch synthase genes determines different respects in amylopectin synthesis through analysis of the SS mutants. In the mutants of rice and Arabidopsis, SSI showed a distinct capacity for the synthesis of chains with DP of 8-12 from DP of 6 to 7 (Fujita *et al.*, 2006; Delvalle *et al.*, 2005). The existence of functional interactions between the starch branching enzymes (SBEII) and the SSI and SSII were reported in classes of amylopectin synthesis in amyloplasts based on the developing wheat endosperm (Tetlow *et al.*, 2008). Alternatively, loss of SSII

specifically also leads to the loss of SSI protein in the granule-bound phase and the effect of this mutation is clearly manifest from the mid-stage of endosperm development in wheat (Kosar-Hashemi *et al.*, 2007).

The isoenzyme of SSI was not reported in the other researches. SSI expressed in the early and middle stage of the development of endosperm (Li *et al.*, 1999). There was conserved sequence in the transit peptide of SSI gene: Lys-Ser-Gly-Gly, which is the site for attached by ADP-Glc (Baba *et al.*, 1993; Tanaka *et al.*, 1995). Soluble starch synthase determined to the chains with degree of polymerization (DP) of 6-15 by the expressing of SS gene and Starch Branching Enzyme (SBE) gene in *E. coli* (Guan and Keeling, 1998). In rice, SSI and SSIIIa were the main soluble starch synthase and SSI had higher activity than SSIIIa, take 70% activity of soluble synthase (Fujita *et al.*, 2006). The similar performances were reported in maize and wheat (Cao *et al.*, 1999; Li *et al.*, 2000). Gene SSI, encoded protein Spg-3 with 75 KDa of molecular weight, was located on 7AS, 7BS and 7DS in wheat (Yamamori and Endo, 1996).

In the present study, in order to understanding the variation of SSI gene and exploiting new genetic resources in *Triticum L.* and *Aegilops*, the partial

sequences of SSI were investigated from *Aeilops tauschii*, *Triticum turgidum*, *Triticum aestivum* and synthetic hexaploid wheat. Phylogenetic analysis was also done for evaluating the relationship between different species and starch synthase genes.

MATERIALS AND METHODS

Materials: A total of four accessions, including AS60, AS2255, SHW-L1 and Chuanmai 32, were used in this study. All the materials were provided by the Germplasm Laboratory of Triticeae Research Institute, Sichuan Agriculture University. Synthetic hexaploid wheat SHW-L1 (AABBDD, *T. aestivum*) was obtained through distant hybridization between accession AS2255 (AABB, *T. turgidum*) and AS60 (DD, *Ae. tauschii*) (Zhang *et al.*, 2004). Variety Chuanmai 32 (AABBDD, *T. aestivum*) was a commercial common wheat cultivar at southwest of China.

Methods: All materials were planted in the field of Sichuan Agricultural University, China. Total RNA was extracted from the new collected seed after pollination 15 days using the total RNA isolation kit (Takara) according to the manufacturer's instruction. To eliminate genomic DNA, extracted RNA was treated with RNAase-free DNase supplement supplied with the kit. RNA quality and purity were tested by UV absorption spectrophotometer and gel electrophoresis. Total RNA was used to synthesize cDNA using the Reverse Transcriptase M-MLV (Takara) and oligo-dT primer according to the manufacturer's instruction. A pair of primers, which were designed based on the sequence AJ292521 (GeneBank), was used as the cloning primers: the forward primer: 5'-TTATTGGAAGACTGGATTACC-3' and the reverse primer: 5'-CACAGCCGTCATCTACTCT-3'. The amplified region includes from the 11th exon to the 15th exon and partial 3'UTR. PCR was performed in a 50 µL volume, containing 1.5 Utaq plus DNA polymerase, 100 ng of each cDNA, 5 µL PCR buffer (supplied with Taq plus DNAPolymerase), 1.5 mM MgCl₂, 100 mM of each dNTP and 150 ng each primer. The reactions were conducted in a PTC-220 (MJ Research, USA) using the following program: 95°C for 3 min, followed by 35 cycles at 94°C for 1 min, at 60°C for 1 min and at 72°C for 2 min and a final extension step of 72°C for 10 min. The PCR products were separated on 1.5% agarose gels. The expected fragments were recovered and cloned into pMD18-T vector (TaKaRa), then transformed into the competent *E. coli* cells (DH5α). Therefore, positive clones of each accession were sequenced by commercial company (Invitrogen) in two directions.

Data analysis: The obtained sequences were compared with known SSI sequences using BLAST (<http://www.ncbi.nlm.nih.gov/BLAST>). Sequences were manually inspected with BioEdit ver 7.0.9 (Hall, 1999). Sequence alignment was completed with ClustalX (Larkin *et al.*, 2007) and DNAMAN 5.2.2 (<http://www.lynnon.com>). Neighbour-joining trees was constructed based on the alignment of all known sequences, including sequences from barley, rice, maize, sorghum and potato respectively, using Kimura 2-parameter model in MEGA4.0 (Saito and Nei, 1987; Tamura *et al.*, 2007).

RESULTS

Variation of sequences of SSI gene: Bands about 750 bp were amplified by PCR from four accessions respectively. After cloning and sequencing these bands, eight sequences were obtained, including one sequence from AS60, two sequences from AS2255, three sequences from Synthetic hexaploid wheat SHW-L1 and two from wheat variety Chuanmai 32. Except from AS2255, the sequences of SSI gene showed the complete same from one sample. Blast in NCBI confirmed all the sequences belonged to the partial SSI gene, suggested that these primers could successfully cloning the SSI gene from *T. aestivum* and *Ae. tauschii*. Analysis of these sequences showed 13 variable nucleotide sites in all five sequences, suggested that there was higher conservative for SSI gene between diploid, tetraploid and hexaploid in *T. aestivum* and *Ae. tauschii*. Interestingly, 10 variable nucleotide sites were observed in sequence AS2255-2 from Synthetic hexaploid wheat SHW-L1, indicated this sequence was clearly distinguishing with other SSI gene.

Only two variable sites, at 47 (Ser/Asn) of sequence AS2255-2 and 141 (Ala/Val) of sequence SHW-L1, were observed between five sequences through alignment of amino acid residue, further suggested that SSI was very high conserve gene in *T. aestivum* and *Ae. tauschii* (Fig. 1). Moreover, the variation of amino acid residues was detected between these five sequence and sequences from *T. aestivum* (CAB99209), *Hordeum vulgare* (CAX51357), *Brachypodium distachyon* (XP003561063), *Zea mays* (AAB99957) and *Oryza sativa* (AAP56350). The results showed the sequences from *Ae. tauschii*, *T. turgidum* and *T. aestivum* were more similar to SSI from common wheat and barley. Sequences from other species, *Brachypodium distachyon*, *Zea mays* and *Oryza sativa* showed more variable amino acid residue, comparing with sequences from *Triticum* L. and *Aegilops*. But there was no variation on two important functional domains, domain II and domain III, between all sequences. These results suggested that although SSI is a very conservative gene, the variation could be observed between different species.

AS60	IGRLDYQKGIDLIKMAIPELMREDVQFVMLGSGDPFIFEGW	40
AS2255-1	IGRLDYQKGIDLIKMAIPELMREDVQFVMLGSGDPFIFEGW	40
AS2255-2	IGRLDYQKGIDLIKMAIPDLMREDVQFVMLGSGDPVFEGW	40
Shw-L1	IGRLDYQKGIDLIKMAIPELMREDVQFVMLGSGDPFIFEGW	40
ChuanMai32	IGRLDYQKGIDLIKMAIPELMREDVQFVMLGSGDPFIFEGW	40
CAB99209	IGRLDYQKGIDLIKMAIPELMREDVQFVMLGSGDPFIFEGW	40
CAX51357	IGRLDYQKGIDLIKMAIPDLMREDVQFVMLGSGDPVFEGW	40
XP003561063	IGRLDYQKGIDLIKLAIIPDLMRDDVQFVMLGSGDPVFEGW	40
AAB99957	IGRLDYQKGIDLIQLIIPDLMREDVQFVMLGSGDPELEDW	40
AAP56350	IGRLDYQKGIDLIKLAIIPDLMRDNIQFVMLGSGDPGFEGW	40
AS60	MRSTESSYKDKFRGWVGFSPVSHRITAGCDILLMPSRFE	80
AS2255-1	MRSTESSYKDKFRGWVGFSPVSHRITAGCDILLMPSRFE	80
AS2255-2	MRSTESSYKDKFRGWVGFSPVSHRITAGCDILLMPSRFE	80
Shw-L1	MRSTESSYKDKFRGWVGFSPVSHRITAGCDILLMPSRFE	80
ChuanMai32	MRSTESSYKDKFRGWVGFSPVSHRITAGCDILLMPSRFE	80
CAB99209	MRSTESSYKDKFRGWVGFSPVSHRITAGCDILLMPSRFE	80
CAX51357	MRSTESSYKDKFRGWVGFSPVSHRITAGCDILLMPSRFE	80
XP003561063	MRSTESSYKDKFRGWVGFSPVSHRITAGCDILLMPSRFE	80
AAB99957	MRSTESSYKDKFRGWVGFSPVSHRITAGCDILLMPSRFE	80
AAP56350	MRSTESSYKDKFRGWVGFSPVSHRITAGCDILLMPSRFE	80
AS60	PCGLNQLYAMQYGTVPVVHGTGGLRDTVETFNPF GAKGEE	120
AS2255-1	PCGLNQLYAMQYGTVPVVHGTGGLRDTVETFNPF GAKGEE	120
AS2255-2	PCGLNQLYAMQYGTVPVVHGTGGLRDTVETFNPF GAKGEE	120
Shw-L1	PCGLNQLYAMQYGTVPVVHGTGGLRDTVETFNPF GAKGEE	120
ChuanMai32	PCGLNQLYAMQYGTVPVVHGTGGLRDTVETFNPF GAKGEE	120
CAB99209	PCGLNQLYAMQYGTVPVVHGTGGLRDTVETFNPF GAKGEE	120
CAX51357	PCGLNQLYAMQYGTVPVVHGTGGLRDTVETFNPF GAKGEE	120
XP003561063	PCGLNQLYAMQYGTVPVVHGTGGLRDTVETFNPF GAKGEE	120
AAB99957	PCGLNQLYAMQYGTVPVVHGTGGLRDTVETFNPF GAKGEE	120
AAP56350	PCGLNQLYAMQYGTVPVVHGTGGLRDTVETFNPF GAKGEE	120
Domain II		Domain III
AS60	GTGWAFSPLTVDKMLWALRTAMSTFREHKPSWEGLMKRG	160
AS2255-1	GTGWAFSPLTVDKMLWALRTAMSTFREHKPSWEGLMKRG	160
AS2255-2	GTGWAFSPLTVDKMLWALRTAMSTFREHKPSWEGLMKRG	160
Shw-L1	GTGWAFSPLTVDKMLWALRTVMSTFREHKPSWEGLMKRG	160
ChuanMai32	GTGWAFSPLTVDKMLWALRTAMSTFREHKPSWEGLMKRG	160
CAB99209	GTGWAFSPLTVDKMLWALRTAMSTFREHKPSWEGLMKRG	160
CAX51357	GTGWAFSPLTVEKMLWALRTAISTFREHKPSWEGLMKRG	160
XP003561063	GTGWAFSPLTVDKMLWALRTAMTTFREHKPSWEGLMKRG	160
AAB99957	GTGWAFAPLITENMLWTLRTAISTYREHKPSWEGLMKRG	160
AAP56350	GTGWAFSPLTIEKMLWALRMAISTYREHKPSWEGLMKRG	160
AS60	TKDHTWDHAAEQYEQIFEWAFVDQPYV	187
AS2255-1	TKDHTWDHAAEQYEQIFEWAFVDQPYV	187
AS2255-2	TKDHTWDHAAEQYEQIFEWAFVDQPYV	187
Shw-L1	TKDHTWDHAAEQYEQIFEWAFVDQPYV	187
ChuanMai32	TKDHTWDHAAEQYEQIFEWAFVDQPYV	187
CAB99209	TKDHTWDHAAEQYEQIFEWAFVDQPYV	187
CAX51357	TKDHTWDHAAEQYEQIFEWAFVDQPYV	187
XP003561063	SKDHTWDHAAEQYEQIFEWAFVDQPYV	187
AAB99957	SKDHTWDHAAEQYEQIFEWAFIDRQPYV	187
AAP56350	SDHTWDHAAEQYEQIFEWAFMDQPYV	187

Fig. 1: Alignment of amino acids residues of partial *SSI* gene, AS60 (*Ae. tauschii*), AS2255-1 and AS2255-2 (*T. turgidum*), SHW-L1(Synthetic hexaploid wheat), Chuanmai 32 (*T. aestivum*) CAB99209 (*Triticum aestivum*), CX51357 (*Hordeum vulgare*), XP003561063 (*Brachypodium distachyon*), AAB99957 (*Zea mays*), AAP56350 (*Oryza sativa*)

Phylogenetic analysis: In order to estimate the relationship of SSI gene between different species, the phylogenetic tree was constructed by neighbor-joining method (Fig. 2). A total of 28 sequences, which included not only the cloning sequences of SSI gene in this study, but also the genes reported in other researches from wheat, barely, rice, maize, maize, sorghum, potato, Arabidopsis and *Brachypodium distachyon*, respectively. Moreover, Granule-bound Starch Synthase (GBSS), SSII, SSIII and SSIV genes were also applied in this phylogenetic analysis. All the sequences could be clustered to four groups. Group I included the new

cloning sequences in this study, the sequences from common wheat and the sequence from sorghum, maize and *Brachypodium distachyon*. Sequences from *Triticum* L. and *Aegilops* were clustered closely than other species in this group. Three sequences from barley, rice and *Ae. tauschii*, respectively, were clustered into group II. Gene GBSSI, GBSSII and the alleles of SSII were clustered into group III. Genes SSI from Arabidopsis and potato were clustered into group IIIIV. This group also included SSIV and SSIII genes from *T. aestivum*, indicated that SSI gene had more similarity with these two genes. Phylogenetic tress further suggested that although high

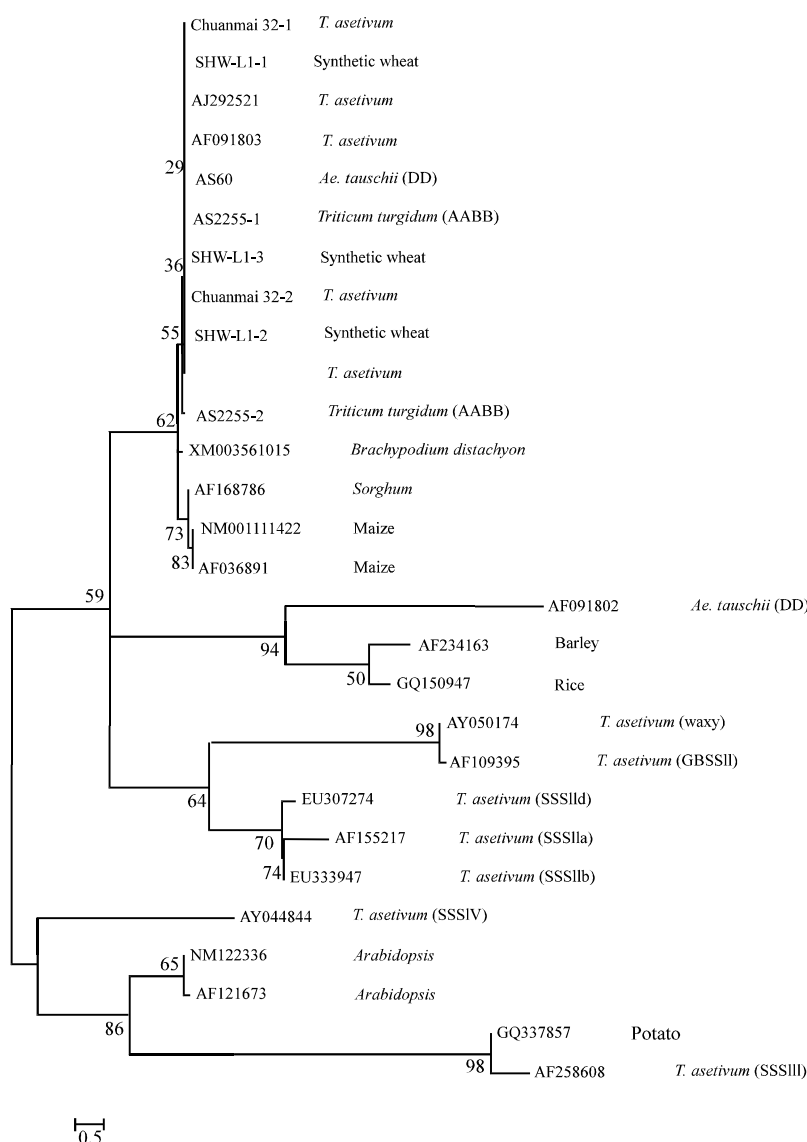


Fig. 2: Neighbour-joining tree based on sequences of SSI gene was constructed by using Kimura 2-parameter model in MEGA 4.0, Bootstrap tree was obtained by 1, 500 repeat calculation

conservative characterization of sequence, the variation of SSI gene could be detected clearly between different species and between different starch synthase genes. These results implied that it was possible to developing the special molecular markers to apply in the improving of wheat starch quality.

DISCUSSION

Both of *Ae. tauschii* and synthetic hexaploid wheat are the very important genetic resources for the breeding of common wheat. *Ae. tauschii*, which usually considered as the donor of D genome in common wheat (McFadden and Sears, 1946; Kihara, 1944) and contains a lot of excellent potential characters (Kimber and Feldman, 1987), as well as the very useful materials to understand the evolution of common wheat (Dudnikov, 1998). Synthetic hexaploid wheat, produced by crossing tetraploid wheat (AABB) with *Ae. tauschii* (DD), has been used as an intermediary for transferring resistance genes from the wild ancestor to cultivated wheat (Del Blanco *et al.*, 2001). Synthetic hexaploids have been reported as having resistance to disease (Lan *et al.*, 1997), tolerant to abiotic stresses and quality (Del Blanco *et al.*, 2001; Kunert *et al.*, 2007). Starch, which accounts for 65-75% of wheat grain dry weight, is also widely used in food industry including bread, noodles, biscuits, cakes and non-food industry including paper, plastic, adhesive, textile, medical and pharmaceutical industry (Sestili *et al.*, 2010). The further improving the quality of starch need to exploiting continuously new gene resources. Starch Synthase (SS) I plays an important role in the series of synthesis of starch of wheat, not only involved in amylopectin synthesis and also in amylose biosynthesis. SSI preferentially synthesizes chains of DP 7-11 by elongating amylopectin short chains of DP 4-7. In present study, eight partial sequences of SSI gene were cloned from *Ae. tauschii*, tetraploid wheat, synthetic hexaploid wheat and common wheat. The results suggested that the variation could be detected between different species, although SSI gene had high conservative characterization. In this study, although the partial sequences of SSI gene were cloned, two substitutions of amino acid residues, Ser/Asn and Ala/Val, were observed in tetraploid AS2255-2 and hexaploid SHW-L1. This result also showed that the variation of SSI gene could be detected in *Triticum* L. and *Aegilops*, suggested that it was possible to developing new molecular markers to exploit SSI gene resources. In addition, the effect of these variations for the function of SSI gene and starch quality should be investigated in these accessions.

Plants contain up to five isoforms of starch synthase that are categorized, according to conserved sequence relationships. These five isoforms include GBSS, SSI, SSII, SSIII and SSIV. SSI exclusively involved in amylopectin biosynthesis. In the present study, the phylogenetic tree showed that these five genes could be clustered clearly into different groups. Although came from different species, most of SSI gene sequences were clustered into one group, indicated SSI gene had higher conservative between different species, especially in *Triticum* L. and *Aegilops*.

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