

# The Structure and Expression of the Wheat Starch Synthase III Gene. Motifs in the Expressed Gene Define the Lineage of the Starch Synthase III Gene Family<sup>1</sup>

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The endosperm of hexaploid wheat (*Triticum aestivum* [L.]) was shown to contain a high molecular weight starch synthase (SS) analogous to the product of the maize *du1* gene, starch synthase III (SSIII; DU1). cDNA and genomic DNA sequences encoding wheat SSIII were isolated and characterized. The wheat SSIII cDNA is 5,346 bp long and contains an open reading frame that encodes a 1,628-amino acid polypeptide. A putative N-terminal transit peptide, a 436-amino acid C-terminal catalytic domain, and a central 470-amino acid SSIII-specific domain containing three regions of repeated amino acid similarity were identified in the wheat gene. A fourth region between the transit peptide and the SSIII-specific domain contains repeat motifs that are variable with respect to motif sequence and repeat number between wheat and maize. In dicots, this N-terminal region does not contain repeat motifs and is truncated. The gene encoding wheat SSIII, designated *ss3*, consists of 16 exons extending over 10 kb, and is located on wheat chromosome 1. Expression of *ss3* mRNA in wheat was detected in leaves, pre-anthesis florets, and from very early to middle stage of endosperm development. The entire N-terminal variable repeat region and the majority of the SSIII-specific domain are encoded on a single 2,703-bp exon. A gene encoding a class III SS from the Arabidopsis genome sequencing project shows a strongly conserved exon structure to the wheat *ss3* gene, with the exception of the N-terminal region. The evolutionary relationships of the genes encoding monocot and dicot class III SSs are discussed.

The synthesis of starch occurs in the plastids of higher plants and involves a series of biosynthetic enzymes, including ADP-Glc pyrophosphorylase, starch synthase (SS), branching enzyme (BE), and debranching enzyme. Multiple isoforms of each activity are present in higher plants. The basic units of starch structure are  $\alpha$ -1,4-linked glucan chains that are linked into higher order structures through  $\alpha$ -1,6 branch points. Starches typically consist of two classes of polymers: amylose, which is a linear or lightly branched molecule (<0.5%  $\alpha$ -1,6 linkages) with a degree of polymerization of 1,000 to 5,000 Glc units, and amylopectin, a much larger (degree of polymerization of  $10^5$ – $10^6$ ) polymer containing frequent  $\alpha$ -1,6 branch points (5%–6%  $\alpha$ -1,6 linkages).

SSs extend  $\alpha$ -1,4 glucan chains by catalyzing the transfer of the glucosyl moiety of ADP-Glc to the non-reducing end of a pre-existing  $\alpha$ -1,4 glucan. So far, four classes of SSs have been found in higher plants: granule-bound SS (GBSS; Kloesgen et al.,

1986; van der Leij et al., 1991; Okagaki, 1992), SSI (Baba et al., 1993; Knight et al., 1998), SSII (Dry et al., 1992; Edwards et al., 1995; Harn et al., 1998), and SSIII (Abel et al., 1996; Marshall et al., 1996; Gao et al., 1998). In cereals, the most comprehensively studied species is maize, where in addition to GBSS, cDNAs encoding SSI, SSIIa, and SSIIb have been isolated. Both cDNA and partial genomic clones derived from the *du1* gene, which encode for the DU1 SS, have been characterized (Gao et al., 1998; Harn et al., 1998; Knight et al., 1998).

The analysis of mutants in a wide range of species demonstrates that GBSS is essential for amylose synthesis (Ball et al., 1996). In addition, GBSS also contributes to the synthesis of amylopectin (Denyer et al., 1996). SSI, SSII, and SSIII are each thought to be primarily involved in amylopectin synthesis, although they may also have non-essential roles in amylose biosynthesis. In maize, two mutations affecting amylopectin biosynthesis are associated with reductions in SS activity. The gene encoding maize SSIIa maps to the same location as the *sugary 2* (*su2*) mutation (Harn et al., 1998). The dull mutation, *du1*, has recently been definitively shown (Gao et al., 1998; Cao et al., 1999) to be caused by a mutation of a structural gene encoding a high  $M_r$  SS present in the soluble phase of the maize amyloplast (Yu et al., 1998). This protein was originally described as SSII

<sup>1</sup> This research was supported by Goodman Fielder (Sydney) and Biogemma (Paris).

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(Boyer and Preiss, 1981), but to avoid confusion with the nomenclature is now defined as DU1 (Cao et al., 1999). *Du1* kernels have a tarnished and glassy endosperm phenotype. The effects of a lesion in *du1* on starch structure are also background dependent but typical effects seen include a decrease in amylopectin content (typically referred to as an increase in apparent amylose content), the presence of intermediate material distinguishable from amylose and amylopectin, and a reduction in chain length distribution of amylopectin (Boyer and Preiss, 1981). The starch granules of *du1* mutants are essentially unaltered in their morphology and properties. In potato, antisense suppression has been used to examine the role of SSIII in starch biosynthesis (Abel et al., 1996). While SSIII expression was decreased to very low levels relative to the wild type, no effects on amylose content or amylopectin chain length distribution were seen, although differences in starch granule morphology and phosphate content were observed (Abel et al., 1996). Differences in the phenotypes of mutants affecting SS expression, and differences in kinetic properties between SS isoforms, suggested that each enzyme tends to contribute to the extension of specific subsets of the available non-reducing ends within amylopectin. Evidence from two similar studies in potato, in which the expression of both SSII and SSIII were reduced by chimeric antisense approaches, provides further support for this hypothesis (Edwards et al., 1999; Lloyd et al., 1999).

In wheat (*Triticum aestivum*), four SS activities have been identified. The 60-kD product of the waxy gene (*wx1*; also known as GBSS) is exclusively found within the starch granule (Rahman et al., 1995). The waxy cDNA has been cloned (Clark et al., 1991) and has been shown to be located on chromosome 7 (A and D genome) and chromosome 4 (B genome; Yamamori and Endo, 1996). A class II SS (SSII; known as the Sgp-1 protein, encoded by the *ss2* gene) is found within the starch granule throughout development and is also present in the soluble phase early in endosperm development (Li et al., 1999a). At mid-endosperm development, two forms of SS could be detected by native PAGE analysis of endosperm crude soluble extracts (Li et al., 1999b). The high electrophoretic mobility form was shown to be SSI (encoded by the *ss1* gene), and the slow electrophoretic mobility form was suggested to be the product of the wheat *ss3* gene and the homolog of the maize high  $M_r$  DU1 encoded by the *du1* gene (Li et al., 1999b).

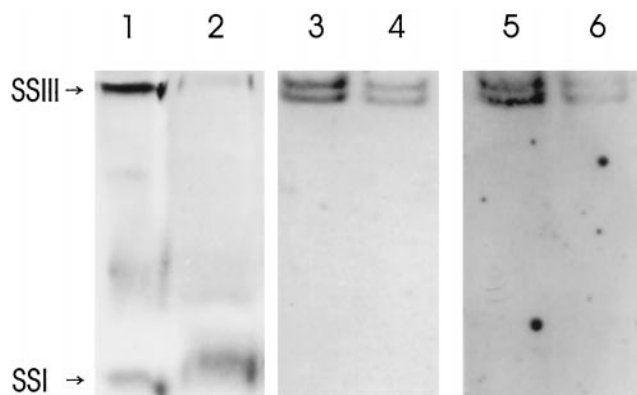
In this paper, we report the cloning of the gene encoding wheat SSIII (designated *ss3*) and the cognate cDNA. We have analyzed the domain structure, exon and intron structure, the chromosome location, and the expression of this gene during wheat development. Comparison of the wheat *ss3* gene with a gene encoding a class III SS from *Arabidopsis* pro-

vides additional information on the degree of structural diversity within the class III SSs.

## RESULTS

### Identification of a Class III SS Activity in Wheat Endosperm Extracts

Zymogram analysis of the soluble fraction of extracts of wheat endosperm (isolated 20 DPA) revealed the presence of two SS activities, a 75-kD SS (wSSI) encoded by the wheat *ss1* gene (Li et al., 1999b), and a second activity with low mobility in non-denaturing PAGE we refer to as wheat SSIII (wSSIII; Fig. 1, lanes 1 and 2). An affinity chromatography step using  $\beta$ -cyclodextrin Sepharose-6B (Pharmacia-Biotech, Piscataway, NJ) was used to rapidly separate proteins with starch-binding capacity away from the majority of the wheat endosperm soluble proteins. Fractions eluted by  $\beta$ -cyclodextrin from the affinity column were electrophoresed in non-denaturing PAGE gels, which were blotted and probed using antibodies to SSIII amino acid sequences. In Figure 1, lanes 3 and 4, the blot has been reacted with antisera (DU1N) raised against the N-terminal region (residues 1–648) of the maize DU1 polypeptide. In Figure 1, lanes 5 and 6, the blot has



**Figure 1.** Zymogram and immunoblotting analysis of wheat endosperm extracts separated by non-denaturing PAGE. Soluble extracts of wheat endosperm were prepared by passage through a  $\beta$ -cyclodextrin Sepharose-6B affinity column and starch-binding proteins eluted with  $\beta$ -cyclodextrin. All starch synthase activities present in the crude extract were bound by the affinity column and were eluted with  $\beta$ -cyclodextrin (data not shown). Lanes 1 and 2 were loaded with 18 and 4  $\mu$ g of column eluate protein, respectively, and SS activity was revealed according to Abel et al. (1996). The 75-kD SSI and low mobility SSIII activities are indicated. Lanes 3 and 4 were immunoblotted with antibodies (DU1N) to the N-terminal region of maize DU1 protein (DU1N antibodies used at 1:5,000 dilution and the film exposed for 30 s prior to development). Lanes 5 and 6 were immunoblotted with affinity purified antibodies ( $\alpha$ KRG9) to a synthetic peptide designed from an amino acid sequence deduced from the nucleotide sequence at the N-terminal of the wSSIII.B3 cDNA ( $\alpha$ KRG9 antibodies were used at 1:100 dilution and the film was exposed for 2 min prior to development). Lanes 3 and 5 were loaded with 4  $\mu$ g of column eluate protein and lanes 4 and 6 with 2  $\mu$ g of eluate protein.

**A Deduced Amino Acid Sequence**

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-> Transit Peptide
1 MEMSLWPRSP LCPRSRQPLV VVRPAGRGL TQPFLMNGRF TRSRTLRCMV
  -> Variable Repeat Region
51 ASSDPPNRKS RRMVPPQVKV ISSRGYTTRL IVEPSNENTE HNNRDEETLD

101 TYNALLSTET AEWTDNREAE TAKADSSQNA LSSSIIGVEVD VADEDILAAD
    RM1.1 RM2.1
151 LTVYSLSSVM KKEVDAADKA RVKEDAFELD LPATLRSVI VDVMDHNGTV
    RM2.2 RM2.3 RM1.2
201 QETLRSVIVD VMDHNGTVQVE TLRSVIVDVM DDAADKARVE EDVFELDLGS
    RM1.3 RM1.4
251 NISSSATVVE LDAVDFVGVF QDKFEATSSG NVNSATVRE VDASDEAGND
    RM1.5
301 QGIFRADLSG NVSSSTTVE VGAVDVAGSI KDRFETDSSG NVTSAPMWD
    RM1.6 RM1.7
351 AIDETVADOD TFRADLSGNA SSCATYREVD DVVDETRSEE ETFAMDLFAS

401 ESGHEKHMVA DYVGEATDEE ETYQQQYVPV SSFMSWDKAI AKTGVSLNPE
    RM3.1 RM3.2 RM3.3 RM3.4
451 LRLVRVEEQG KVNFSDKKDL SIDDLPGONO SIIGSYKODK SIADVAGPTO
    RM3.5 RM3.6 RM3.7 RM3.8 RM3.9
501 SIFGSSKQHR SIVAFPKONO SIVSVTEOKO SIVGFRSODL SAVSLPKONV

551 PIVGTSTREGQ TKQPVPVDRQ DALYVNGLEA KEGDHTSEKT DEDALHVKFN

601 VDNVLRKHQA DRTQAVEKKT WKKVDEEHLY MTEHQKRAE QGMVVNEDEL

651 SITEIGMGRG DKIQHVLSEE ELSWSEDEVQ LIEDDGQYEV DETSVSVNVE
    RM4.1
  -> SSIII Specific Region
701 QDIQGSPODV VDPQALKVML QELAEKNYSM RNKLFFVPEV VKADSVIDLX
    RM5.1
751 LNRDLTALAN EPDVVTKGAF NGWKWRLTFE RLHKSPLGGV WWSCKLYTPK

801 EAYRLDFVFF NGRTVYENNG NNDFCIGIEG TMNEDLFEDF LVKEKQRELE
    RM6.1 RM6.2
851 KLAMEEAERR TOTEORRRK BARAADEAVR QAQAEIETK KKKLOSMLS
    RM4.2
901 ARTCVDNLWY IEASTDTRGD TIRLYNRRNS RPLAHSTEIW MHGGYNNWTD
    RM5.2
951 GLSIVESFVK CNDKDGDWY ADVIPPEKAL VLDVWFADGP AGNARNYDNN
    RM6.3
1001 ARQDFHAILP NNNVTEEGFW AQEEQNIYTR LLOERRREKRE TMKRKAERSA
    RM4.3
1051 NIKAEMKAKT MRRFLLSQKH IVYTEPLETR AGTTVDVLYN PSNTVLNGKS
    RM5.3
1101 EGWFRCSFNL WMHSSGALPP QKMWKSGDGP LLKATVDVPP DAYMDFVFS
    CM1
  ->
1151 EWNEEDGIYDN RNGMDYHIPV SDSIETENYM RIIHIAVEMA PVAKVGGGLGD
    Catalytic Domain
1201 VVTSLSRAIQ DLGHTVEVIL PKYDCLNQSS VKDLHLHYQSF SWGTEIKVW

1251 VGRVEDLTVY FLEPQNGMFG VGCVYGRNDD RRFGFFCHSA LEFILQNEFS

1301 PHIIHCHDWS SAPVAWLYKE HYSQSRMAST RVVFTIHNLE FGAHYIGKAM
    CM2 CM3
1351 TYCDKATTVS PTYSRDVAGH GAIAPHREKF YGILNGIDPD IWDPYTDNFI

    CM4 CM5
1401 PVPYTCENVV EGKRAAKRAL QOKFGLQQT VPIVGIITRL TAQKGIHLIK
    CM6
1451 HAIHRTLESN GHVVLLGSAP DHRIQGDPCR LADALHGVIY GRVKLVLTVD
    CM7 CM8
1501 EPLSHLIYAG SDFIIVPSIF EPGCLTOLVA MRYGSIPIVR KTGGLHDTVF

1551 DVDNDKDRAR SLGLEPNGFS FDGADNSGVD YALNRAIGAW FDARDWFHSL

1601 CKRVMEQDWS WNRPALDYIE LYHAARKF*
    
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**B Comparison of Repeat Motif Sequences**

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Motif 1
RM1.1 164 VDAADKARVKEDAFELDLP 183
RM1.2 231 DDAADKARVEEDVFELDLSGNISSSATVVE 260
RM1.3 261 LDAVDFVGVFVQDKFEATSSGNVNSATVRE 290
RM1.4 291 VDASDEAGNDQGITFRADLSGNVFSSTTVE 320
RM1.5 321 VGAVDEAGSTKDRFETDSSGNVTSAPMWD 350
RM1.6 351 AIDETVADODTFRADLSGNASSCATYREVD 380
RM1.7 381 DVVDETRSEEETFAMDLFAS 402

Motif 2
RM2.1 185 TLRSVIVDVMHNGTVQVE 202
RM2.2 203 TLRSVIVDVMHNGTVQVE 220
RM2.3 221 TLRSVIVDVM 230

Motif 3
RM3.1 467 KKDLSIDDLR 476
RM3.2 477 QONOSTIGSY 486
RM3.3 487 KQDKSIADVA 496
RM3.4 497 GPTQSTFGSS 506
RM3.5 507 KOHRSIIVAF 516
RM3.6 517 KONQSTIVSVT 526
RM3.7 527 BQKQSTIVGFR 536
RM3.8 537 SODLSAVSLP 546
RM3.9 547 KONVPIVGT 556

Motif 4
RM4.1 742 KADSVLDLYLNRDLTALANEPDVVVKGAFCNW 773
RM4.2 917 TRGDTIRLYNRRNSRPLAHSTEIWMHGGYNNW 948
RM4.3 1069 RAGTTVDVLYNPSNVVLNGKSEGWFRCSENLW 1111

Motif 5
RM5.1 781 DLGGVWWSCKLYIPKEAYRLDFVFNDRGT 814
RM5.2 958 DKDGDWVYADVIPPEKALVLDVWFADGPA 991
RM5.3 1121 SGDGPLLKAIVDVPDAYSMDVFESEWEE 1154

Motif 6
RM6.1 859 RRQTTEORRRKBARAAD 876
RM6.2 882 QAQAEIETKKKKLOSMLS 899
RM6.3 1029 QERREKETMKRKAERSA 1050
    
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**Figure 2.** Wheat SSIII.B3 deduced amino acid sequence analysis and comparison (A) deduced amino acid sequence showing domains (as defined in Table I) and repeat motifs (from RM1–RM6, as defined by intra-sequence analysis using the program DOTPLOT; see Fig. 4). Conserved motifs, as defined in Cao et al. (1999), are marked as CM1 to CM8. B, Comparison of the amino acid sequences of repeat motifs (RM1–RM6). At each position, the most frequent residue is shown with a double underline, and residues similar to this residue, according to Dayhoff and Orcutt (1979), are single underlined. At positions where no residue is more frequent than any other, no residues are underlined.

mobility as the low electrophoretic mobility SS band revealed by activity staining (Fig. 1, lanes 1 and 2). We conclude that the low mobility SS is a class III SS analogous to the product of the maize *du1* gene. The higher mobility immunoreactive band may be an inactive degradation product of the active species.

**Isolation of cDNA Sequences Encoding Wheat SSIII**

Screening of a cDNA library from hexaploid wheat with a 478-bp probe, wSSIIIp2 (see “Materials and Methods”) yielded eight cDNA clones that were purified and partially sequenced. Three highly homologous (>96% identity using the BestFit program, Genetics Computer Group, Madison, WI; Devereaux et al., 1984) yet distinct classes of cDNA encoding wSSIII were obtained. Of these cDNAs, one cDNA

been reacted with affinity purified antibodies ( $\alpha$ KRG9) raised against a synthetic peptide designed from a deduced amino acid sequence of the N-terminal region of the wheat wSSIII.B3 cDNA (see next section). In both immunoblotting experiments, two polypeptides of identical electrophoretic mobility were immunoreactive. The band with lower electrophoretic mobility migrates with precisely the same

was demonstrated to contain a full-length SSIII open reading frame (ORF; wSSIII.B3, 5,346-bp insert, GenBank accession no. AF258608). The other two classes of cDNAs were represented by the partial cDNAs wSSIII.B1 (3,664 bp, corresponding to the region from 1,690–5,346 of wSSIII.B3) and wSSIII.B2 (4,143 bp, corresponding to the region from 1,204–5,346 of the comparable region of wSSIII.B3), respectively.

An ORF was found in clone wSSIII.B3 from an ATG codon starting at nucleotide 29 of the cDNA and extending to a stop codon ending at nucleotide 4,915. The amino acid sequence deduced from this ORF is composed of 1,628 amino acid residues (Fig. 2), with a predicted molecular mass of 183 kD. A putative transit peptide cleavage site was identified by neural network analysis by the program ChloroP between amino acid residues 67 and 68 (Emanuelsson et al., 1999). The molecular mass of the mature protein is therefore predicted to be 176 kD.

### Comparison with Other SSs

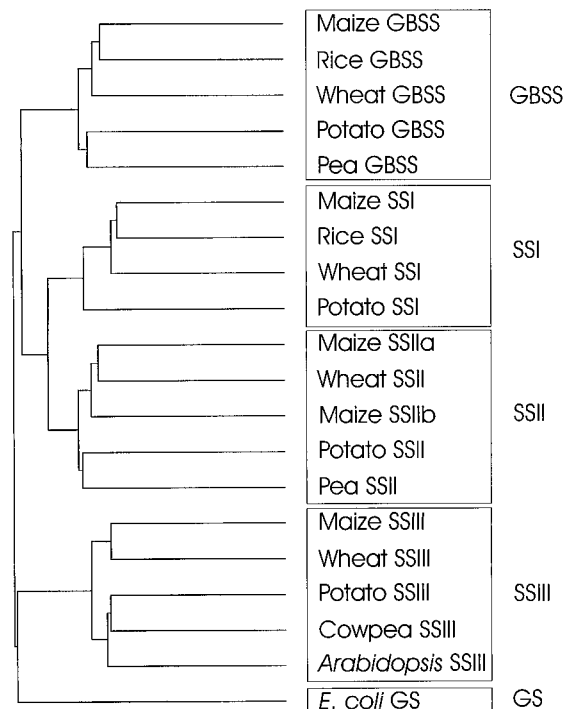
Relationships among the amino acid sequences of plant SSs were analyzed with the program PILEUP in the Genetics Computer Group suite of programs (Fig. 3). The SS groups were separated into four classes: GBSS, SSI, SSII, and SSIII. The wSSIII.B3 sequence is found in the class III SS group which clusters more closely with glycogen synthase from *Escherichia coli* than with other plant SSs. Within all four classes of SSs, the monocot sequences and dicot sequences form distinct subclasses.

### Domain Organization of wSSIII

Gao et al. (1998) concluded that the maize SSIII sequence contained four distinct regions: a putative transit peptide region, a *du1*-specific N-terminal region, a central region homologous to other class III SSs, and a C-terminal region that contained the catalytic domain. The subsequent availability of sequences for genes encoding cowpea (GenBank accession no. AJ225088) and Arabidopsis SSIII (GenBank accession no. AC007296), and the wheat sequence presented here, provides a basis for further analysis of these domain regions. Table I shows definitions of the domain regions of the genes encoding SSIII from wheat, maize, potato, cowpea, and Arabidopsis based on the following analysis of these sequences.

### Transit Peptide

The precise transit peptide cleavage sites for SSIII proteins have not been experimentally defined. Putative transit peptide cleavage sites were identified using the ChloroP neural network analysis of the 100 amino acids at the N terminus of each sequence (Emanuelsson et al., 1999). The sites identified by this analysis were similar in wheat and maize, but di-



**Figure 3.** Relationships between the primary amino acid sequences of plant SSs and *E. coli* glycogen synthase. The dendrogram was generated by the program PILEUP (Devereaux et al., 1984). The C-terminal 1,000-amino acid residues from wheat SSIII, potato SSIII, and maize SSIII (DU1) were used in the analysis as inclusion of the entire amino acid sequences for these genes and could not be analyzed by the PILEUP (a Genetics Computer Group program). The sequences used are: maize GBSS (Kloesgen et al., 1986), rice GBSS (Okagaki, 1992), wheat GBSS (Clark et al., 1991), potato GBSS (van der Leij et al., 1991), pea GBSS (Dry et al., 1992), maize SSI (Knight et al., 1998), rice SSI (Baba et al., 1993), wheat SSI (Li et al., 1999a), potato SSI (GenBank accession no. Y10416), wheat SSIIA (Li et al., 1999b), maize SSIIa and SSIIb (Harn et al., 1998), pea SSII (Dry et al., 1992), potato SSII (Edwards et al., 1995), maize SSIII (DU1; the product of the *du1* gene; Gao et al., 1998), wheat SSIII (wSSIII.B3; this paper), potato SSIII (Abel et al., 1996), cowpea SSIII (GenBank accession no. AJ225088), Arabidopsis SSIII (GenBank accession no. AC007296), and *E. coli* glycogen synthase (GS; Kumar et al., 1986).

verged in the dicots. Figure 1, lanes 5 and 6, provides evidence supporting a cleavage site prior to residue 68 because the antibody cross-reacting with wheat SSIII in these studies was raised against a synthetic peptide extending over the region from residue 69 to 85 of the wheat SSIII amino acid sequence (Fig. 2).

### N-Terminal Variable Repeat Region

PILEUP analysis (Devereux et al., 1984) was used to quantify the percentage of amino acid identity between various regions of the SSIII amino acid sequences (Table I). This analysis showed that while there was high sequence identity in the SSIII-specific and C-terminal catalytic domains between wheat and maize, there was low conservation of identity in the region extending from the putative transit peptide to

**Table I.** Domains within SSIII-deduced amino acid sequences

Species	Parameter	Putative Transit Peptide	N-Terminal Region	SSIII-Specific Region	Catalytic Domain
Wheat	Position	1–67	68–723	724–1,193	1,194–1,629
	No. of residues	67	656	470	436
Maize	Position	1–69	70–770	771–1,239	1,240–1,674
	No. of residues	69	701	469	435
	Identity <sup>a</sup>	52%	37%	73%	83%
Potato	Position	1–90	91–324	325–793	794–1,230
	No. of residues	90	234	469	437
	Identity <sup>a</sup>	30%	21%	58%	74%
Cowpea	Position	1–80	81–241	242–710	711–1,147
	No. of residues	80	162	469	437
	Identity <sup>a</sup>	26%	17%	58%	76%
Arabidopsis	Position	1–20	21–115	116–590	591–1,025
	No. of residues	20	96	475	435
	Identity <sup>a</sup>	20%	12%	56%	72%

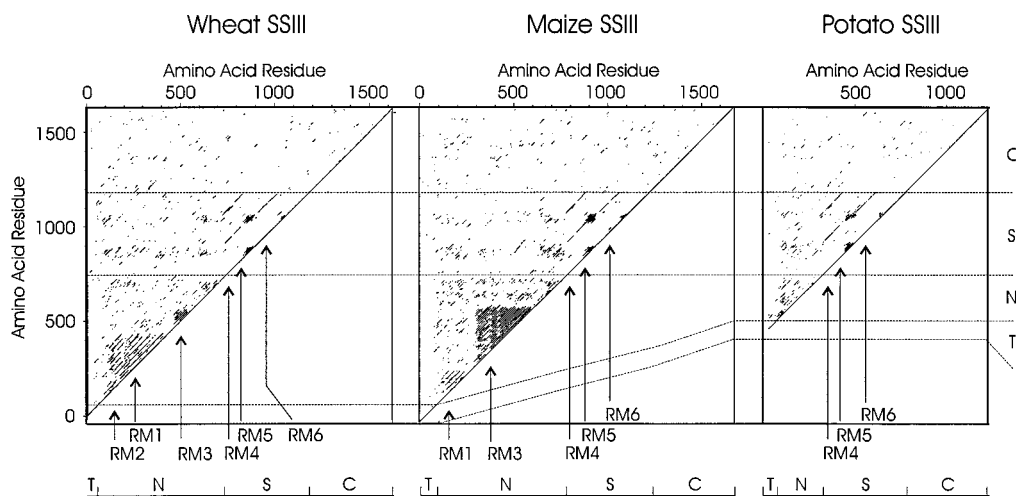
<sup>a</sup> Identity with Wheat SSIII.

the SSIII-specific region. The DOTPLOT program (Devereaux et al., 1984) was used to identify repeated regions of amino acid sequence similarity within the deduced amino acid sequences and Figure 4 shows the output of the DOTPLOT analysis for wheat, maize, and potato. The sequences of six repeat regions identified in the wheat SSIII.B3 sequence using DOTPLOT are marked in Figure 2A and the wheat repeat motifs are aligned and compared in Figure 2B. The positions of the repeats are also shown in Figure 5.

The outputs for cowpea and Arabidopsis were essentially identical to potato and are not shown. The monocot and dicot N-terminal region sequences (defined in Table I) show marked differences in both length and

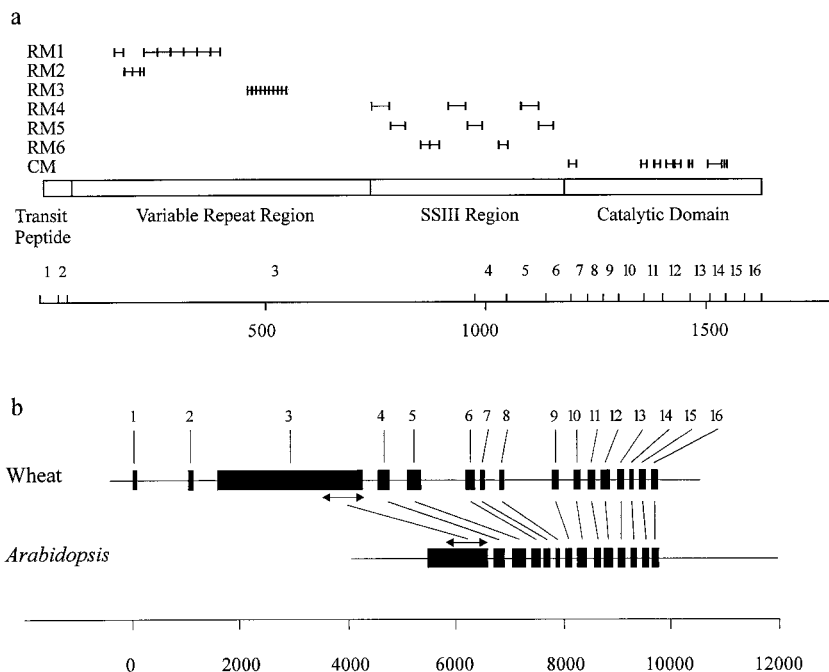
homology, and there is only very weak evidence of repeats in the dicot sequences (Fig. 4). We, therefore, refer to the region between the putative transit peptide and the SSIII-specific region as the N-terminal variable repeat region in the monocot sequences and as the N-terminal region in the dicot sequences.

Within the two available monocot N-terminal variable repeat regions, there are also marked differences between the wheat and maize sequences. Both sequences contain a set of six repeated amino acid blocks of approximately 30 amino acid residues designated as the RM1 motif repeats in Figure 2 (see also Figs. 4 and 5a). Gao et al. (1998) considered these repeats in maize DU1 to form part of three larger repeats each containing two repeated motifs. In



**Figure 4.** Analysis of repeat regions within the deduced amino acid sequences of SSIII from wheat, maize, and potato. The DOTPLOT program from the Genetics Computer Group package was used to examine each of the deduced amino acid sequences for repeated motifs within each gene by comparing each sequence against itself, residue by residue, and assigning a score based on an amino acid similarity index, using a compare window of 30 and a stringency parameter of 11 (Devereux et al., 1984). Repeated regions are revealed through this analysis as lines parallel to the diagonal. Domains are indicated on the x axis and on the right hand side: C, catalytic domain; S, SSIII-specific region; N, N-terminal region; T, transit peptide, and repeat motifs (RMs) are shown below each dot plot. Both the domain and repeat motif sequences are defined in Figure 2.

**Figure 5.** a, Domain structure of the wheat SSIII amino acid sequence showing the contribution of each exon to the amino acid sequence and the position of repeat (RM) and conserved (CM) motifs. The size scale is indicated below the sequence in amino acid residues. b, The exon and intron structure of the wheat *ss3* and a gene encoding Arabidopsis SSIII. Exons are indicated as black boxes and the introns are shown as solid lines. The size scale is indicated below the gene in base pairs. Double-headed arrows show homologous regions present at the 3' ends of exon 3 of wheat *ss3* and exon 1 of a gene encoding Arabidopsis SSIII.



wheat, an additional block of three repeat motifs, the RM2 repeats, are found after the first occurrence of the RM1 motif (RM1.1) and before RM1.2 (Fig. 2). Whereas RM2.1 and RM2.2 are perfect repeats at the amino acid level, the third RM2.3 repeat is truncated.

In maize, a set of eighteen 10-amino acid repeats, designated as the starch BE repeats, were found in the N-terminal region (Gao et al., 1998). However, in wheat SSIII, just nine analogous repeats (designated here as the RM3 repeats) are present. This difference in repeat structure between wheat and maize is illustrated in the intra-sequence DOTPLOT program analysis shown in Figure 4.

### SSIII-Specific Region

Comparison of the maize and potato SSIII sequences by Gao et al. (1998) identified a central region showing homology between these genes, but no homology to other SSs. Comparison of wheat, maize, potato, cowpea, and Arabidopsis sequences extends this analysis and confirms the presence of this SSIII-specific region in each gene.

The intra-sequence DOTPLOT program analysis of repeat regions within the genes encoding SSIII identified a set of three repeated units of amino acid similarity in each gene (Fig. 4). Whereas the amino acid sequence identity is low between repeats, these regions were identified on the basis that they were repeats in which amino acid similarity was conserved. This suggests that, although there was little selection for specific amino acids in these regions, there was pressure for conservation of amino acid functionality. These regions of amino acid similarity are shown in Figure 2 as repeat motifs RM4, RM5,

and RM6, respectively. It is striking that there is conservation of repeated units in each gene, which indicates the retention of repeat architecture across the evolutionary distance that separates monocots and dicots. On the basis of this comparison, we define the SSIII region (residues 771–1,239 of maize SSIII) to be somewhat larger than the definition (residues 770–1,028) used by Gao et al. (1998) to accommodate all of the repeated motifs. Table I shows the definition of this region and the identity to the wheat SSIII amino acid sequence for each gene encoding SSIII.

### C-Terminal Catalytic Domain

Previous studies of plant SSs (Harn et al., 1998; Li et al., 1999a) have identified the presence of a highly conserved C-terminal region that contains all of the residues required for catalysis (Cao et al., 1999). The wheat SSIII sequence contains a domain at the C terminus that contains each of eight highly conserved regions present (designated CM1–8) in SSs (Cao et al., 1999; Figs. 2 and 5a) beginning from the first conserved region, KVGGLGDVVVTSLSRAIQDLGHTVEV.

### Isolation of Wheat *ss3* Sequences

Screening of a genomic library from the D-genome donor of wheat, *Triticum tauschii* identified 10 clones which hybridized to the wSSIII.B3 PCR fragment, wSSIIIp1. Sequence analysis of these clones demonstrated that all of the clones were derived from one gene and their sequences were subsequently assembled into one contiguous fragment to determine the gene structure. We define the homologous set of

wheat genes encoding wheat SSIII as the wheat *ss3* genes.

The contiguous sequence containing the *ss3* gene is 10-kb long and contains 16 exons separated by 15 introns (Table II; GenBank accession no. AF258609). The exon and intron structure of the gene is shown in Figure 5b and is marked by the presence of an unusually large exon, exon 3, spanning 2,703 bp. Exon 3 extends from the proposed transit peptide, through the N-terminal variable repeat region, and into the SSIII-specific region. Exon 3 has no significant homology with sequences other than with other genes encoding SSIII in the GenBank databases using FASTA analysis (Devereaux et al., 1984).

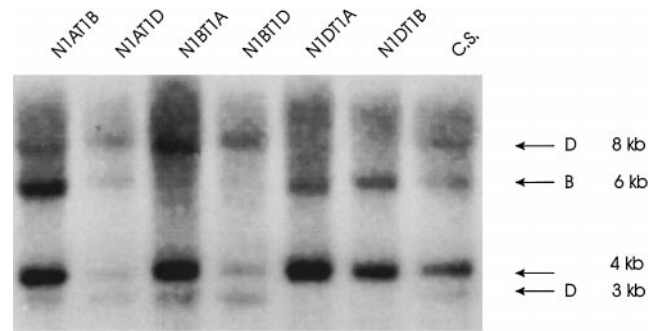
A gene encoding Arabidopsis SSIII was detected from GenBank (using the program BLAST) when wSSIII.B3 was used as an inquiry sequence. The Arabidopsis sequence is from the BAC clone T18B16 from Arabidopsis chromosome 4 (GenBank accession no. AL021687).

The inferred exon and intron structure of the gene encoding Arabidopsis SSIII is also given in Figure 5b, along with the wheat exon and intron structure. Comparison of the exon structures of wheat *ss3* and the Arabidopsis gene showed that the exon structure at the C terminus of wheat *ss3* (exons 4–16) is highly similar to Arabidopsis (exons 2–14; Fig. 5b; Table II). However, wheat *ss3* contains three exons in the 5' region of the gene (exons 1–3) and the gene encoding Arabidopsis SSIII has just one exon in the 5' region of the gene (exon 1). Figure 5b also shows that wheat *ss3* contains significantly larger introns than the Arabidopsis gene.

**Table II.** Location of exons in wheat *ss3* and a gene encoding Arabidopsis SSIII

Exon <sup>a</sup>	Wheat <i>ss3</i> <sup>b</sup>	Gene Encoding Arabidopsis SSIII <sup>b</sup>	Identity	Start Site <sup>c</sup>	End Site <sup>c</sup>
			%		
1	126	–	–	–27 <sup>d</sup>	99
2	64	–	–	1,057	1,120
3	2,703	1,108	53.9	1,589	4,291
4	218	215	59.5	4,550	4,767
5	271	274	67.5	5,104	5,374
6	176	176	65.9	6,149	6,324
7	108	108	67.6	6,439	6,546
8	110	110	71.8	6,793	6,902
9	103	106	66.9	7,798	7,900
10	171	171	69.0	8,165	8,335
11	129	129	68.2	8,461	8,589
12	183	183	70.5	8,678	8,860
13	132	132	65.9	8,978	9,109
14	112	112	69.6	9,206	9,317
15	129	129	70.2	9,408	9,536
16	529	130	72.1	9,619	10,147 <sup>e</sup>

<sup>a</sup> Intron positions are given in Genbank records for the wheat *ss3* and a gene encoding Arabidopsis SSIII (a gene encoding Arabidopsis SSIII, accession no. AC007296; wheat *ss3*, to be submitted).  
<sup>b</sup> Exon length.    <sup>c</sup> Positions given refer to the wheat *ss3* gene.  
<sup>d</sup> Nucleotide 1 is the start of the ORF.    <sup>e</sup> Nucleotide 9,746 is the end of the ORF.



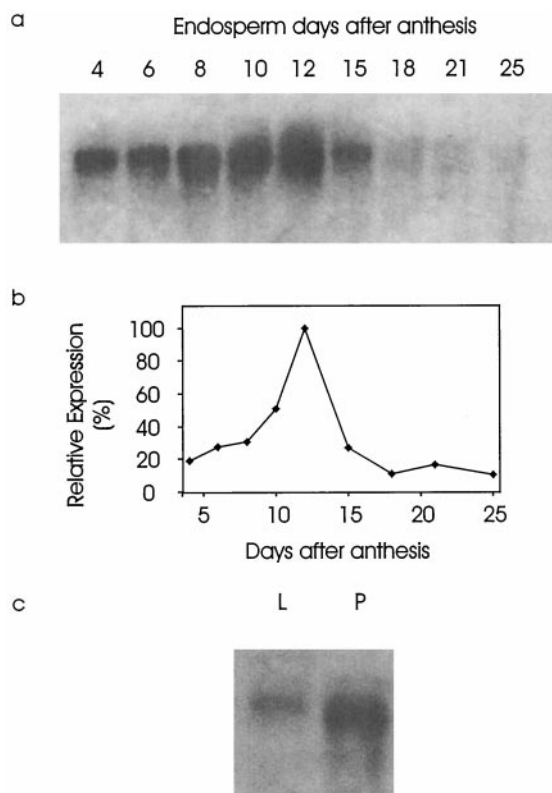
**Figure 6.** Chromosome localization of the wheat *ss3* genes by Southern-blot analysis. Total wheat genomic DNAs were isolated from the leaves of cv Chinese Spring homologous group-1 compensating nullisomic and tetrasomic lines of wheat. N1AT1B, Nullisomic 1A and tetrasomic 1B; N1AT1D, nullisomic 1A and tetrasomic 1D; N1BT1A, nullisomic 1B and tetrasomic 1A; N1BT1D, nullisomic 1B and tetrasomic 1D; N1DT1A, nullisomic 1D and tetrasomic 1A; N1DT1B, nullisomic 1D and tetrasomic 1B; and C.S., hexaploid wheat cv Chinese Spring. The genomic DNA was digested with *DraI* restriction enzyme and probed with wSSIIIp3 fragment from wSSIII.B3. The DNA fragments (from 3–8 kb) from B and D genomes are indicated by arrows. No specific fragment from the A genome was detected.

**The Promoter of the Wheat *ss3* Gene**

The wheat *ss3* upstream region contains a putative TATA box at the nucleotides –353 to –356. It also has the sequence (TGGAAAAG) at nucleotides –1,339 to –347, which is similar to the EM-box motif (TGTA-AAG; Ford et al., 1985) associated with endosperm-specific expression. Comparison of the sequences between –1 and –400 of wheat *ss1* and *ss3* promoters gives a low percentage of identity (approximately 37.9%). However, a sequence (CCACCGTCCG) similar to the motif 2 [CCG(T/C)CCGTCCG] of wheat *ss1* described by Li et al. (1999a) is also found at nucleotides –299 to –309.

**Wheat *ss3* Genes Are Located on the Group 1 Chromosomes**

Four bands (approximately 3, 4, 6, and 8 kb) were detected when the 5' part of wSSIII.B3 (nucleotides 24–460) was used as a probe for Southern blotting (Fig. 6). Analysis of nullisomic/tetrasomic wheat lines showed that the 3- and 8-kb bands were encoded by the D genome and that the 6-kb band was encoded by the B genome. A 4-kb band could not be assigned to any chromosomes using this analysis and this band may represent either a fragment that is common to the *ss3* genes from more than one genome, or may contain several fragments from the gene, rendering assignment impossible using this enzyme and probe combination. Evidence from PCR assays of polymorphisms among the *ss3* genes supports the localization of an *ss3* gene on chromosome 1A (data not shown).



**Figure 7.** RNA-blot analysis from wheat using wheat SSIII cDNA sequences as probe. Total RNAs were isolated from endosperm of wheat cv Gabo and probed with wSSIIIp1 fragment from the wSSIII.B3 cDNA. a, Endosperm mRNA of wheat cv Gabo. The ages of the endosperm tissues in number of DPA are given above the lanes. b, Quantification of the RNA expression data is shown in a. The RNA blot was scanned and the intensity of each band was digitally recorded. The same blot was stripped and probed with an 18S RNA probe, and the signal from bands at each stage was recorded. The wheat *ss3* expression at each age was normalized against the 18S data and the maximum expression level defined as 100% expression. c, L refers to leaf RNA, and P refers to RNA from pre-anthesis florets.

#### RNA-Blot Hybridization Analysis of Wheat *ss3* mRNA Expression

The expression of wheat *ss3* mRNA was analyzed by the RNA-blotting analysis. The total RNA purified from the endosperm of wheat cv Gabo was used for the study of the expression of wheat *ss3* mRNA during endosperm development, using a probe that does not cross-react with *wx*, *ss1*, or *ss2* mRNA under the probe hybridization conditions used. The accumulation of wheat *ss3* mRNA is seen at 4 DPA, and the level of expression increases from 4 to 12 DPA and then declines from 15 to 25 DPA (Fig. 7, a and b). mRNA for class III SS is also expressed in the leaf and in pre-anthesis florets (Fig. 7c).

#### DISCUSSION

Immunoblotting and zymogram analysis demonstrates that there are at least four prominent SS ac-

tivities in wheat endosperm: the GBSS, SSI (Li et al., 1999b), SSII (Li et al., 1999a), and an SS with low electrophoretic mobility in non-denaturing gels (Li et al., 1999b; Fig. 1, lanes 1 and 2). Two independent lines of evidence (Fig. 1) demonstrate that the low mobility SS activity is encoded by a class III SS gene. Firstly, antibodies to the N-terminal region of the maize DU1 protein (a region not homologous to other SSs) shows two cross-reacting bands of low electrophoretic mobility in non-denaturing PAGE of wheat endosperm soluble extracts. The slower migrating band of the pair is aligned precisely with the low electrophoretic mobility SS band. Secondly, an antibody raised against a synthetic peptide designed from the deduced amino acid sequence of the wheat *ss3* sequence cross-reacts with two bands of identical electrophoretic mobility to the two bands that react with the antibody to the maize DU1 protein. We conclude, therefore, that the slowest mobility band reacting with these antibodies is an SSIII-type SS and that the higher mobility immunoreactive band may be an inactive degradation product.

In wheat, GBSS is entirely granule-bound (Denyer et al., 1995; Rahman et al., 1995), SSI is partitioned between the granule and the soluble fraction (Li et al., 1999b), and SSII is predominantly granule-bound, although it is present in the soluble fraction early in endosperm development (Li et al., 1999a). In contrast, the SSIII protein in this study has not been found to be associated with the starch granule and is exclusively found within the soluble fraction of the endosperm (data not shown).

The work of Gao et al. (1998) and Cao et al. (1999) demonstrates that in maize, the DU1 protein is encoded by the *du1* gene. The availability of three additional class III SS sequences, from wheat (this study), cowpea (GenBank accession no. AJ225088), and Arabidopsis (GenBank accession no. AC007296) allows for the extension of observations made here regarding the structure of the genes encoding SSIII from maize (Gao et al., 1998; Cao et al., 1999). The general domain structure of the genes encoding SSIII is conserved among genes from both monocots and dicots. The monocot genes differ from the dicot sequences in having an extended region at the N terminus containing variable repeat motifs. In contrast to maize, wheat contains an additional repeated motif in the N-terminal variable repeat region (motif RM2 in Figs. 2 and 5a) and contains nine repeats of motif RM3 (Figs. 2 and 5a) compared with 18 in maize. The conservation of sequence between this repeat motif and starch BE is less obvious than in maize and it appears unlikely that the specific nature or number of the repeats is essential for functionality.

Comparison of genes encoding SSIII using the intra-sequence DOTPLOT program analysis demonstrated that there are three repeated motifs within the SSIII-specific region of each of the five gene sequences available. These motifs are recognized not



through direct perfect repeats of regions of sequence identity, but rather, through imperfect repeats containing identical residues embedded in stretches of residues showing conservation of amino acid residue functional groups. Strikingly, these repeated regions were found in each of the genes encoding SSIII and the arrangement of the repeats is conserved. The specific contributions of either the N-terminal variable region or the central SSIII-specific regions to the catalytic properties of the enzyme are unknown. However, the C-terminal region of maize SSIII expressed in *E. coli* was capable of catalysis, demonstrating that residues essential for catalysis are not located outside of the C-terminal conserved domain (Cao et al., 1999). Additional work is required to establish the specific contribution of the variable repeat and SSIII-specific regions to the catalytic properties of SSIII.

The wheat *ss3* gene contains 16 exons and one exon alone accounts for approximately 50% of the ORF. This exon begins just after the transit peptide and contains the N-terminal variable repeat region and much of the SSIII-specific region. In contrast, in the gene encoding Arabidopsis SSIII, a single smaller exon (exon 1) encodes the transit peptide, the truncated N-terminal region, and the corresponding region of the SSIII-specific region found in the exon 3 of wheat. It is interesting to note that, without exon 3 of wheat *ss3*, or exon 1 of the gene encoding Arabidopsis SSIII, the encoded proteins would be comparable in size to the deduced amino acid sequences of the *ss1* and *ss2* genes of wheat, suggesting that a single insertion event introduced an additional exon into the gene encoding SSIII, predating the divergence of the monocots and dicots, that has modulated the activity or interactions of the SSIII enzyme in ways that are beneficial and therefore retained.

In the remainder of the gene encoding SSIII, extending from exon 4 to 16, the exon and intron arrangement of the wheat and Arabidopsis genes are extremely well conserved. Although extensive changes in intron sequences have occurred, the conservation of exon sequences and the actual positioning of introns suggests that the lineage of genes encoding SSIII predates the separation of monocotyledonous and dicotyledonous plants. This observation suggests that the SSIII gene lineage was present in early dicotyledonous members of the *Magnoliiflorae* that most likely gave rise to monocotyledonous plants (Dahlgren et al., 1985). The observation, furthermore, suggests that the SSIII gene lineage is at least 100 million years old, based on the suggested separation of the monocotyledon and dicotyledon groups of plants in the Cretaceous period (Barlow, 1981; Clegg, 1990). In contrast to this high degree of conservation, the N-terminal variable repeat region (exons 1–3 in wheat) is highly variable, as illustrated in the wheat, maize, and Arabidopsis comparison discussed above. Comparison of the exon and intron structures of the wheat

*wx1*, wheat *ss1*, and wheat *ss3* genes shows that neither the exon-intron boundaries, nor the location of conserved motifs in the C-terminal catalytic domain within exons, is conserved across these genes (data not shown), suggesting that the divergence of the genes for the SS isoforms is ancient.

The *ss3* gene has been shown to be located on chromosome 1 of the wheat genome, in contrast to the genes encoding other SSs, *wx1*, *ss1*, and *ss2* which are all located on the short arm of chromosome 7 (Yamamori and Endo, 1996; Li et al., 1999a, 1999b). The independent chromosomal location of *Ss3* away from the chromosome 7 SSs is consistent with the possibility, raised above, that SSIII genes represent a distinct gene lineage.

The expression of all *ss1*, *ss2*, and *ss3* mRNAs were detected in the endosperm of wheat. However, the expression of wheat *ss3* mRNA in the endosperm is earlier than that of wheat *ss1* and *ss2* mRNAs (Li et al., 1999a, 1999b). The expression of *ss2* mRNA was also detected in the leaves and pre-anthesis florets of wheat, however, the expression of the *ss1* gene was endosperm specific.

The conservation of the number and properties of SS isoforms across higher plants suggests that each isoform has a specific role in the synthesis of starch. The isolation of mutants affecting GBSS, SSII, and SSIII suggests that the activities of each of these cannot be fully complemented by the activities of one or more of the remaining isoforms. At the biochemical level, the differences between isoforms in kinetic properties that define their individual roles are still unresolved, largely because of the difficulties of generating a suitable range of defined substrates. The role of the SSIII class of SSs has been best defined in maize through the analysis of the *du1* mutants and in potato through antisense down regulation of the SSIII gene. Analysis of SS sequences using PILEUP shows that the SSIII group of gene sequences (Fig. 3) cluster closer to bacterial glycogen synthases than to other higher plant SSs. One point of similarity between SSIII and glycogen biosynthetic enzymes is that SSIII is primarily located in the soluble fraction of cereal endosperm, unlike GBSS, SSI, and SSII which are either entirely or partially granule-bound. This may indicate that SSIII plays a role in the synthesis of short chains of amylopectin which are in turn substrates for other SSs (SSI, SSII, and GBSS), similar to the role of bacterial glycogen synthases in synthesizing the short external chains of glycogen. Additional research is required to establish whether there are more functional similarities between SSIII and glycogen synthases.

In wheat, the hexaploid nature of the genome prevents the ready identification of analogous mutations in *ss3*. The specific contribution of SSIII to starch biosynthesis in wheat may be probed in the future by the identification and combination of mutations in the genes encoding SSIII in each genome or through

suppression of SSIII expression using transgenic approaches. Null backgrounds for GBSS (Yamamori et al., 1994) and SSII (Yamamori, 1998) are available to examine epistatic interactions between *ss3* and *wx*, and *ss3* and *ss2*, respectively. The differences in starch granule structure and functionality between wheat and maize suggest that it is difficult to predict the precise impact of suppression of wheat *ss3* on either starch structure or on the technological use of the wheat grain.

## MATERIALS AND METHODS

### Plant Material

Genetic stocks of hexaploid bread wheat (*Triticum aestivum* cv Chinese Spring) with various chromosome additions and deletions were kindly supplied by Dr. E. Lagudah (Commonwealth Scientific and Industrial Research Organization Plant Industry, Canberra, Australia) and derived from stocks described in Sears and Miller (1985). The hexaploid wheats cv Hartog and cv Gabo were grown in controlled growth cabinet conditions (18°C day and 13°C night, with a photoperiod of 16 h). Wheat leaves, florets prior to anthesis, and endosperm collected over the grain filling period were immediately frozen in liquid nitrogen and stored at -80°C until required.

### Extraction and Purification of SSIII of Wheat

Developing endosperm (4 g), 12 to 16 DPA, were isolated and ground in a mortar and a pestle with 3 volumes of extraction buffer (20 mM Tris-HCl, pH 7.5, 5 mM DTT, and 1 mM pefabloc) at 4°C. The homogenate was then centrifuged at 10,000g for 20 min at 4°C and the supernatant was applied to a 4-mL  $\beta$ -cyclodextrin Sepharose-6B column, equilibrated with 10 mL of extraction buffer. The column was washed with 50 mL of buffer at a flow rate of 1 mL min<sup>-1</sup> and then eluted with 5 mL of the same buffer containing 1% (w/v)  $\beta$ -cyclodextrin. Fractions of 1 mL were collected and immediately tested for SS activity using a zymogram procedure (Abel et al., 1996).

### Gel Electrophoresis and Immunoblotting

Non-denaturing PAGE was carried out using 8% (w/v) gels and activity staining was performed as previously described (Abel et al., 1996). Antisera (DU1N), raised against an N-terminal region of the maize DU1 protein, were a generous gift of Dr. Alan M. Myers (Iowa State University, Ames) and are described by Cao et al. (1999). A rabbit polyclonal antiserum (designated  $\alpha$ KRG9) was raised against an N-terminal synthetic peptide of the deduced wheat SSIII protein. Prior to immunization, the peptide VISSRGYTTTRLIVEPS was linked to KLH (Pierce Chemical, Rockford, IL) via a glycine-cysteine spacer/linker dipeptide (added during peptide synthesis to the C terminus of the peptide) using the heterobifunctional cross-linking reagent MBS (Pierce). An affinity column of the peptide linked to Sulfolink coupling gel (Pierce) was used

to purify antibodies specific for the peptide from the rabbit polyclonal serum using standard techniques (Harlow and Lane, 1999). Immunoblotting was carried out according to Li et al. (1999a).

### Isolation of Total RNA from Wheat

Total RNA was isolated from the leaf, floret, and endosperm tissues of wheat essentially as described in Rahman et al. (1999). RNA was quantified both by UV absorption and by separation in 1.4% (w/v) agarose-formaldehyde gels which were then visualized under UV light after staining with ethidium bromide.

### cDNA Library Construction

A total of 5  $\mu$ g of mRNA, isolated from hexaploid wheat (cv Hartog) endosperm tissue at 8, 10, and 12 DPA, was used for the construction of a cDNA library. The ZAP-cDNA Gigapack III Gold cloning kit (Stratagene, La Jolla, CA) was used for cDNA synthesis and library construction following the protocol supplied. The inserts were directionally cloned using *Xho*I and *Eco*RI with the poly(A) tail at the *Xho*I site. Screening of the amplified library, using 25,000 plaque-forming units per membrane, and the isolation of the cDNAs in the pBluescript vector was also carried out following the manufacturer's protocol.

### Isolation of SSIII cDNA Clones

PCR primers (wSS3pa, 5'-GGAGGTCTTGGTGATGT-TGT-3'; and wSS3pb, 5'-CTTGACCAATCATGGC AATG-3') were used to amplify sequences from wheat endosperm cDNA. A 347-bp fragment (designated wSSIIIp1 and corresponding to the region from 3,614–3,940 of the wSSIII.B3 sequence) was amplified and cloned. These primers were designed based on the conserved nucleotide sequences of the maize *du1* cDNA (wSS3pa, nucleotides 3,843–3,862; wSS3pb, nucleotides 4,170–4,189) and the potato SSIII cDNA (wSS3pa, nucleotides 2,528–2,547; wSS3pb, nucleotides 2,858–2,874). The primers bind to regions encoding the polypeptides GGLGDVV (wSS3pa) and HCHDWS (wSS3pb) in each cDNA. Sequence analysis of wSSIIIp1 indicated that it had a high identity with analogous regions of the genes encoding SSIII from maize (84.4%) and potato (71.8%). wSSIIIp1 was used as a probe in a hybridization screen of  $1 \times 10^5$  plaques from the wheat cDNA library described above. A 3-kb cDNA was identified from among eight positive cDNA clones. This clone was sequenced and a 478-bp fragment from the 5' end of this cDNA (designated as wSSIIIp2 and corresponding to the region from 2,465–2,943 of wSSIII.B3) was amplified by PCR (using primers wSS3pc, 5'-GCACGGTCT ATGAGAACAATGGC-3'; and wSSpd, 5'-TCTGCATACCACCAATCGCCG-3'), and used as a probe to rescreen  $2 \times 10^5$  plaques from the wheat cDNA library yielding eight cDNAs containing a full-length SSIII ORF.

## Genomic DNA- and RNA-Blot Analysis

DNA was isolated and analyzed using standard techniques. Approximately 20  $\mu\text{g}$  of DNA was digested with the restriction enzyme *DraI*, separated on a 1% (w/v) agarose gel, and transferred to reinforced nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA) and hybridized with an  $\alpha$ - $^{32}\text{P}$ -labeled cDNA probe (wSSIIIp3) corresponding to nucleotides 24 to 460 of wSSIII.B3 cDNA. wSSIIIp3 was generated by PCR using the primers (wSS3pe, 5'-GGGAAATGGAGATGT CTCTCTGG-3'; and wSS3pf, 5'-TTCATCCGCCAC ATCCAATTCC-3'). The hybridization and wash conditions were as described in Rahman et al. (1997).

For RNA-blot analysis, 10  $\mu\text{g}$  of total RNA was separated in a 1.4% (w/v) agarose-formaldehyde gel, transferred to a nylon membrane (Hybond N<sup>+</sup>, Amersham, Buckinghamshire, UK), and hybridized with cDNA probe wSSIIIp1 at 42°C in hybridization buffer (Rahman et al., 1999). The cDNA fragment, wSSIIIp1, was labeled with the Rapid Multiprime DNA probe labeling kit (Promega, Madison, WI).

## Construction and Screening of Genomic Library

The genomic library used in this study, prepared from *T. tauschii* var. *strangulata* (GenBank accession no. CPI 110799), was previously described in Rahman et al. (1997). Positive plaques in the genomic library were selected from among  $1 \times 10^5$  plaques as those hybridizing with wSSIIIp1. Hybridization was carried out in 50% (w/v) formamide, 6 $\times$  SSPE, 0.5% (w/v) SDS, 5 $\times$  Denhardt's, and 1.7  $\mu\text{g}/\text{mL}$  salmon sperm DNA at 42°C for 16 h, then washed three times with 2 $\times$  SSC containing 0.1% (w/v) SDS at 65°C for 1 h per wash.

By analysis with cDNA probes (wSSIIIp3, defined above, and wSSIIIp4, generated by restriction enzyme digestion of wSSIII.B3 using *Bam*HI and *Xho*I and extending from nucleotides 5,050–5,346), two genomic DNA clones were selected and used for sequence analysis. Purified DNA from these clones were digested with either *Bam*HI or *Bgl*II and cloned into the *Bam*HI site of pJKKm (Kirschman and Cramer, 1988).

## DNA Sequencing and Analysis

DNA sequencing was performed using the automated ABI system with dye terminators as described by the manufacturer (Applied Biosystems International, Foster City, CA). DNA sequences were analyzed using the Genetics Computer Group suite of programs (Devereaux et al., 1984).

## ACKNOWLEDGMENTS

The authors thank Dr. Alan M. Myers for the generous donation of antibodies to the maize DU1 protein. We thank Tanya Phongkham for assistance in the preparation of wheat mRNA blots and cDNA libraries. We also thank Drs. Tony Ashton and Kym Turnbull for the critical reading of and helpful suggestions for this manuscript. The authors

express their gratitude to Dr. C.B. Hudson for his support for this research program.

Received November 23, 1999; accepted February 21, 2000.

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