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RESEARCH PAPER

Expression profiling of genes involved in starch synthesis in sink and source organs of rice

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Abstract

A comprehensive analysis of the transcript levels of genes which encode starch-synthesis enzymes is fundamental for the assessment of the function of each enzyme and the regulatory mechanism for starch biosynthesis in source and sink organs. Using quantitative real-time RT-PCR, an examination was made of the expression profiles of 27 rice genes encoding six classes of enzymes, i.e. ADPglucose pyrophosphorylase (AGPase), starch synthase, starch branching enzyme, starch debranching enzyme, starch phosphorylase, and disproportionating enzyme in developing seeds and leaves. The modes of gene expression were tissue- and developmental stage-specific. Four patterns of expression in the seed were identified: group 1 genes, which are expressed very early in grain formation and are presumed to be involved in the construction of fundamental cell machineries, *de novo* synthesis of glucan primers, and initiation of starch granules; group 2 genes, which are highly expressed throughout endosperm development; group 3 genes, which have transcripts that are low at the onset but which rise steeply at the start of starch synthesis in the endosperm and are thought to play essential roles in endosperm starch synthesis; and group 4 genes, which are expressed scantly, mainly at the onset of grain development, and might be involved in synthesis of starch in the pericarp. The methodology also revealed that the defect in the cytosolic *AGPase small subunit2b* (*AGPS2b*) transcription from the *AGPS2* gene in endosperm sharply enhanced the expressions of endosperm and leaf plastidial *AGPS1*, the endosperm cytosolic *AGPase large subunit2* (*AGPL2*), and the leaf plastidial *AGPL1*.

Key words: Endosperm, real-time RT-PCR, rice, starch, transcriptome.

Introduction

Starch is the end-product of photosynthesis in source tissues and is stored as energy reserves in sink tissues. Starch has two major components, the basically linear α-polyglucan amylose and the branched α-polyglucan amylopectin. The α-1,4-glucosidic link chains of both amylose and amylopectin are elongated by the addition of the glucose moiety from ADPglucose, which is synthesized by ADPglucose pyrophosphorylase (AGPase) from glucose-1-P, to the non-reducing end of the α-glucan acceptor molecule. The elongation reactions for the α-1,4-chains of amylose and amylopectin are distinctively catalysed by a starch granule-bound form of starch synthase (GBSS) and a soluble form of starch synthase (SS), respectively. Amylopectin has a much more defined structure called 'tandem-cluster structure' than glycogen, because it is composed of tandem-linked clusters (approximately 9–10 nm each in length) where linear α-1,4-glucan chains are regularly branched via α-1,6-glucosidic linkages, as opposed to the glycogens of bacteria and animals which have a more randomly branched structure (Thompson, 2000). The distinct structure of amylopectin contributes to the crystalline organization of the starch granule (Gallant

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et al., 1997). Variation of the cluster fine structure is considered to cause variations in starch functional properties between species, tissues, and genetic backgrounds.

Amylose is synthesized by AGPase and GBSS while amylopectin is synthesized by the co-ordinated actions of AGPase, SS, starch branching enzyme (BE), and starch debranching enzyme (DBE). Disproportionating enzyme (DPE) and phosphorylase (PHO) are generally considered to be involved in starch degradation, but recent studies suggest that both DPE and PHO may play some parts in starch biosynthesis, although the precise mechanisms of their roles are unclear (Colleoni *et al*., 1999; Ball and Morell, 2003; Tetlow *et al*., 2004). A greater complexity in plant starch biosynthesis is that, although the α-1,4- and α-1,6-glucosidic linkages of glycogen in bacteria and animals can possibly be synthesized by a single form of glycogen synthase and glycogen branching enzyme, those of amylopectin are formed by multiple types of SS (SSI, SSII, SSIII, and SSIV), BE (BEI and BEII), and DBE [isoamylase (ISA) and pullulanase (PUL)]. In addition, several types of these enzymes have multiple isoforms, the number of which is plant species specific. Of these examined so far, each isoform of SS, BE, and DBE plays a distinct role in amylopectin biosynthesis (Nakamura, 2002). Therefore, analysis of the expression patterns of individual genes is important to understand the features of tissue-specific and developmental stage-specific starch biosynthesis.

Metabolic pathways involved in starch biosynthesis are different between source and sink tissues. In addition to the structural difference in amylopectin and starch granules, the carbon of starch is derived from fructose-6-P in the Calvin–Benson cycle in photosynthetic tissues, while in sink tissues it is derived from sucrose, which is translocated from source tissues through the phloem. Therefore, sucrose must be metabolized to sugar-P or ADPglucose in the cytoplasm, and either or both of these compounds are translocated into the amyloplasts via the compound-specific hexose monophosphate translocator or the ADGglucose translocator, respectively (James *et al*., 2003).

The completed genome sequences of the model plants *Arabidopsis* and rice make it possible to predict the total number of rice genes involved in starch biosynthesis. Recently, Smith *et al*. (2004) analysed the diurnal changes in the transcriptome of genes encoding the starch-metabolizing enzyme in the leaves of the dicot *Arabidopsis* to reveal both transcriptional and post-transcriptional regulation of starch metabolism. However, their analyses were confined to leaves. As far as is known, no similar study has been performed in monocots such as cereals, although transcript levels of some classes of enzyme in rice plants such as *SS* (Hirose and Terao, 2004; Jiang *et al*., 2004; Dian *et al*., 2005; Suzuki *et al*., 2005) and *BE* genes (Mizuno *et al*., 2001; Suzuki *et al*., 2005) have been reported.

In the present study, changes in transcripts of all the known genes encoding six classes of enzymes presumably involved in starch synthesis, including AGPase, SS plus GBSS, BE, DBE, PHO, and DPE in developing rice endosperm were measured by quantitative real-time PCR, and the results were compared with their transcript levels in the leaves. The present observations revealed that the pattern of gene expression is gene specific, depending on each gene of each class during endosperm development, and that the genes which are highly expressed in the endosperm are quite different from genes that are markedly expressed in the leaf. The dramatic effects of the rice *shrunken* mutation defect in the cytosolic *AGPase small subunit2b* (*AGPS2b*) transcription from the *AGPS2* gene on the expressions of other *AGPase* genes are also reported.

Materials and methods

Plant material

Rice plants (*Oryza sativa* ssp. *japonica* cv. Nipponbare) were grown in the field in plastic pots filled with paddy field soil. After heading, rice plants were transferred to a greenhouse. For seed sampling, spikelets were marked on the flowering day and harvested at 1, 2, 3, 5, 7, 10, 15, and 20 d after flowering (DAF). For leaf analysis, rice plants (cv. Nipponbare) were grown in a paddy field and 1 month thereafter, three leaf blades were taken from individual plants: the uppermost expanding leaf (young) and the two below (the upper one considered as a fully developed leaf and the lower one as an old leaf). Once severed from the plant, all seed and leaf samples were immediately frozen in liquid nitrogen and stored at −80 °C until use.

For mutant analysis, *Oryza sativa* ssp. *japonica* cv. Kinmaze (wild type) and its *shrunken2* mutant EM22 were grown in a paddy field. For total RNA extractions, the fully developed leaf and developing seeds were harvested when heads hung down, immediately frozen in liquid nitrogen, and stored at −80 °C until use.

RNA extraction and cDNA preparation

Total RNAs were isolated from seed endosperms using the method of Chang *et al*. (1993). First-strand cDNA was synthesized with 5 μg total RNA using SuperScript II RNase H^- Reverse Transcriptase (Invitrogen) and oligo-dT_{13} primer.

Total RNA from leaves of cv. Nipponbare, and seeds and leaves of cv. Kinmaze and its *shrunken* mutant EM22 were extracted with the RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. The extracts were treated with RNase-free DNase (Qiagen) to completely remove contaminating genomic DNA. Two micrograms of leaf or seed total RNA were used for first-strand cDNA synthesis with the iScript™ cDNA Synthesis Kit (Bio-Rad).

Quantitative real-time RT-PCR

Aliquots of the first-strand cDNA mixtures corresponding to 5 ng of total RNA served as the templates for quantitative real-time RT-PCR analysis with the Quantitect™ SYBR Green PCR Kit (Qiagen). Reactions were carried out on an iCycler (Bio-Rad) according to the manufacturers' protocols. The gene-specific primers used for quantitative PCR are listed in Table 1. To optimize PCR conditions for each primer set, annealing temperature, PCR efficiency, and standard curve were examined. The specificity of the PCR amplification was checked with a melt curve analysis (from 55 °C to 94 °C) following the final cycle of the PCR. To verify the specificity of each primer set, their amplification products were cloned in the pGEM T-Easy vector (Promega) and sequenced in an ABI 3100 Automated DNA Sequencer (Applied Biosystems).

Table 1. *Target genes for analysis of expression profile*

Enzyme	Gene name	EC no.	Acc. no.	Chr.	Primer sequence	Amplicon size (bp)	Other name (reference)
ADP-glucose pyrophosphorylase small subunit 1	*OsAGPS1*	2.7.7.27	AK073146	9	[F] GTGCCACTTAAAGGCACCATT [R] CCCACATTTCAGACACGGTTT	97	
ADP-glucose pyrophosphorylase small subunit 2a	*OsAGPS2a*	2.7.7.27	AK071826	8	[F] ACTCCAAGAGCTCGCAGACC [R] GCCTGTAGTTGGCACCCAGA	147	
ADP-glucose pyrophosphorylase small subunit 2b[a]	*OsAGPS2b*	2.7.7.27	AK103906	8	[F] AACAATCGAAGCGCGAGAAA [R] GCCTGTAGTTGGCACCCAGA	186	*Shrunken* (Kawagoe *et al.*, 2005)
ADP-glucose pyrophosphorylase large subunit 1	*OsAGPL1*	2.7.7.27	D50317	5	[F] GGAAGACGGATGATCGAGAAAG [R] CACATGAGATGCACCAACGA	140	
ADP-glucose pyrophosphorylase large subunit 2[a]	*OsAGPL2*	2.7.7.27	U66041	1	[F] AGTTCGATTCAAGACGGATAGC [R] CGACTTCCACAGGCAGCTTATT	96	
ADP-glucose pyrophosphorylase large subunit 3	*OsAGPL3*	2.7.7.27	AK069296	3	[F] AAGCCAGCCATGACCATTTG [R] CACACGGTAGATTCACGAGACAA	131	
ADP-glucose pyrophosphorylase large subunit 4	*OsAGPL4*	2.7.7.27	AK121036	7	[F] TCAACGTCGATGCAGCAAAT [R] ATCCCTCAGTTCCTAGCCTCATT	77	
Starch synthase I	*OsSSI*	2.4.1.21	D16202	6	[F] GGGCCTTCATGGATCAACC [R] CCGCTTCAAGCATCCTCATC	279	
Starch synthase IIa	*OsSSIIa*	2.4.1.21	AF419099	6	[F] GCTTCCGGTTTGTGTGTTCA [R] CTTAATACTCCCTCAACTCCACCAT	54	SSII-3 (Hirose *et al.*, 2004; Jiang *et al.*, 2004)
Starch synthase IIb	*OsSSIIb*	2.4.1.21	AF395537	2	[F] TAGGAGCAACGGTGGAAGTGA [R] GTGAACGTGAGTACGTGACCAAT	89	SSII-2 (Hirose *et al.*, 2004; Jiang *et al.*, 2004)
Starch synthase IIc	*OsSSIIc*	2.4.1.21	AF383878	10	[F] GACCGAAATGCCTTTTTCTCG [R] GGGCTTGGAGCCTCTCCTTA	256	SSII-1 (Hirose *et al.*, 2004)
Starch synthase IIIa	*OsSSIIIa*	2.4.1.21	AY100469	8	[F] GCCTGCCCTGGACTACATTG [R] GCAAACATATGTACACGGTTCTGG	334	SSIII-2 (Hirose *et al.*, 2004)
Starch synthase IIIb	*OsSSIIIb*	2.4.1.21	AF432915	4	[F] ATTCCGCTCGCAAGAACTGA [R] CAACCGCAGGATAACGGAAA	224	SSIII-1 (Hirose *et al.*, 2004)
Starch synthase IVa	*OsSSIVa*	2.4.1.21	AY100470	1	[F] GGGAGCGGCTCAAACATAAA [R] CCGTGCACTGACTGCAAAAT	237	SSIV-1 (Hirose *et al.*, 2004)
Starch synthase IVb	*OsSSIVb*	2.4.1.21	AY373258	5	[F] ATGCAGGAAGCCGAGATGTT [R] ACGACAATGGGTGCCAAGAT	75	SSIV-2 (Hirose *et al.*, 2004; Jiang *et al.*, 2004)
Granule-bound starch synthase I	*OsGBSSI*	2.4.1.21	X62134	6	[F] AACGTGGCTGCTCCTTGAA [R] TTGGCAATAAGCCACACACA	218	*Waxy* (Okagaki, 1992)
Granule-bound starch synthase II	*OsGBSSII*	2.4.1.21	AY069940	7	[F] AGGCATCGAGGGTGAGGAG [R] CCATCTGGCCCACATCTCTA	246	
Starch branching enzyme I	*OsBEI*	2.4.1.18	D11082	6	[F] TGGCCATGGAAGAGTTGGC [R] CAGAAGCAACTGCTCCACC	191	
Starch branching enzyme IIa	*OsBEIIa*	2.4.1.18	AB023498	4?	[F] GCCAATGCCAGGAAGATGA [R] GCGCAACATAGGATGGGTTT	128	
Starch branching enzyme IIb	*OsBEIIb*	2.4.1.18	D16201	2	[F] ATGCTAGAGTTTGACCGC [R] AGTGTGATGGATCCTGCC	261	*Amylose-extender* (Nishi *et al.*, 2001)
Starch debranching enzyme: Isoamylase I	*OsISA1*	3.2.1.68	AB093426	8	[F] TGCTCAGCTACTCCTCCATCATC [R] AGGACCGCACAACTTCAACATA	132	*Sugary-1* (Kubo *et al.*, 1999)
Starch debranching enzyme: Isoamylase II	*OsISA2*	3.2.1.68	AC132483	5	[F] TAGAGGTCCTCTTGGAGG [R] AATCAGCTTCTGAGTCACCG	170	
Starch debranching enzyme: Isoamylase III	*OsISA3*	3.2.1.68	AP005574	9	[F] ACAGCTTGAGACACTGGGTTGAG [R] GCATCAAGAGGACAACCATCTG	100	
Starch debranching enzyme: Pullulanase	*OsPUL*	3.2.1.41	AB012915	4	[F] ACCTTTCTTCCATGCTGG [R] CAAAGGTCTGAAAGATGGG	202	

Table 1. (*Continued*)

Enzyme	Gene name	EC no.	Acc. no.	Chr.	Primer sequence	Amplicon size (bp)	Other name (reference)
Starch phosphorylase L	*OsPHOL*	2.4.1.1	AK063766	3	[F] TTGGCAGGAAGGTTTCGCT [R] CGAAGCCTGAAGTGAACTTGCT	66	
Starch phosphorylase H[a]	*OsPHOH*	2.4.1.1	AK103367	1	[F] CACCAAGACGAAGCTCATCAAG [R] TTCACTCGTTGCTGGGTTCTC	126	
Disproportionating enzyme I	*OsDPE1*	2.4.1.25	AP004306	7	[F] TCTGTCGCTTGAACTTACAGAA [R] TTTGGCTGCAAGTATTGTTCT	77	
Disproportionating enzyme II[a]	*OsDPE2*	2.4.1.25	AK067082	7	[F] CAAGTACACCACAAGACCAGCAA [R] CGTCCAACAGCGAATCCAAT	107	

[a] Cytosolic type.

Determination of mRNA copy number

The copy number of mRNA was determined by a procedure based on that recommended by Applied Biosystems (http://www.appliedbiosystems.com/support/apptech/). In this procedure, the mass of a single plasmid template containing the target sequence was calculated and this mass was equated to one copy of the target gene sequence. The purified plasmid template was then quantified and serially diluted in TE to obtain plasmid solutions that differed by several orders of magnitude. Aliquots were used as templates for quantitative real-time PCR. Data were plotted to generate the standard curve. Plotting the values obtained by quantitative real-time PCR from any sample against this standard curve yields the approximate copy number of the target gene in the sample. In this study, the gene sequence copy number was equated to the mRNA copy number. The amount of mRNA synthesized by each gene was normalized as copy number per nanogram of total RNA according to Bustin (2000) and Gachon *et al*. (2004).

Identification of the members of starch-metabolizing enzyme gene families with DNA database search and computer analysis

To identify the members of each of the gene classes involved in starch biosynthesis, the databases of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) were searched for general entries of nucleotide sequences, and the KOME (Knowledge-based Oryza Molecular biological Encyclopedia) database of the National Institute of Agrobiological Sciences (http://cdna01.dna.affrc.go.jp/cDNA/) for full-length cDNAs. Multiple sequence alignment analyses for the deduced amino acid sequences were carried out using the CLUSTAL W program (http://clustalw.genome.jp/).

Results

Phylogenetic relationships among the genes for starch synthesis in rice, maize, Arabidopsis*, and potato*

The present study focused on the genes encoding six classes of enzymes, namely AGPase, SS (and GBSS), BE, DBE (ISA and PUL), PHO, and DPE (Table 1). For each enzyme class, the phylogenetic relationships among the rice genes and their corresponding homologues in another widely studied monocot maize (*Zea mays*) and the model dicot *Arabidopsis thaliana* were examined. In cases when genes corresponding to those of rice were absent in maize, the homologous genes from potato (*Solanum tuberosum*) were included in the analysis. The results of the comparisons are presented as dendrograms in Fig. 1. In all cases, the rice genes were phylogenetically more related to those of maize than *Arabidopsis*. These data support the current view that the evolution of the starch biosynthetic genes in monocots occurred after the divergence of the monocots from the dicots.

The dendrograms indicate that rice *AGPS1* and *AGPS2* were more recently evolved than the older *AGPL3* and *AGPL4*, both of which have homologues reported in *Arabidopsis* (*AtAPL1* and *AtAPL2*, respectively) but not in maize (Fig. 1A). The biggest class of starch-synthesizing genes, the starch synthase (SS), was phylogenetically separated into five subgroups corresponding to the five subclasses of *SS* genes: *SSI*, *SSII*, *SSIII*, *SSIV*, and *GBSS*

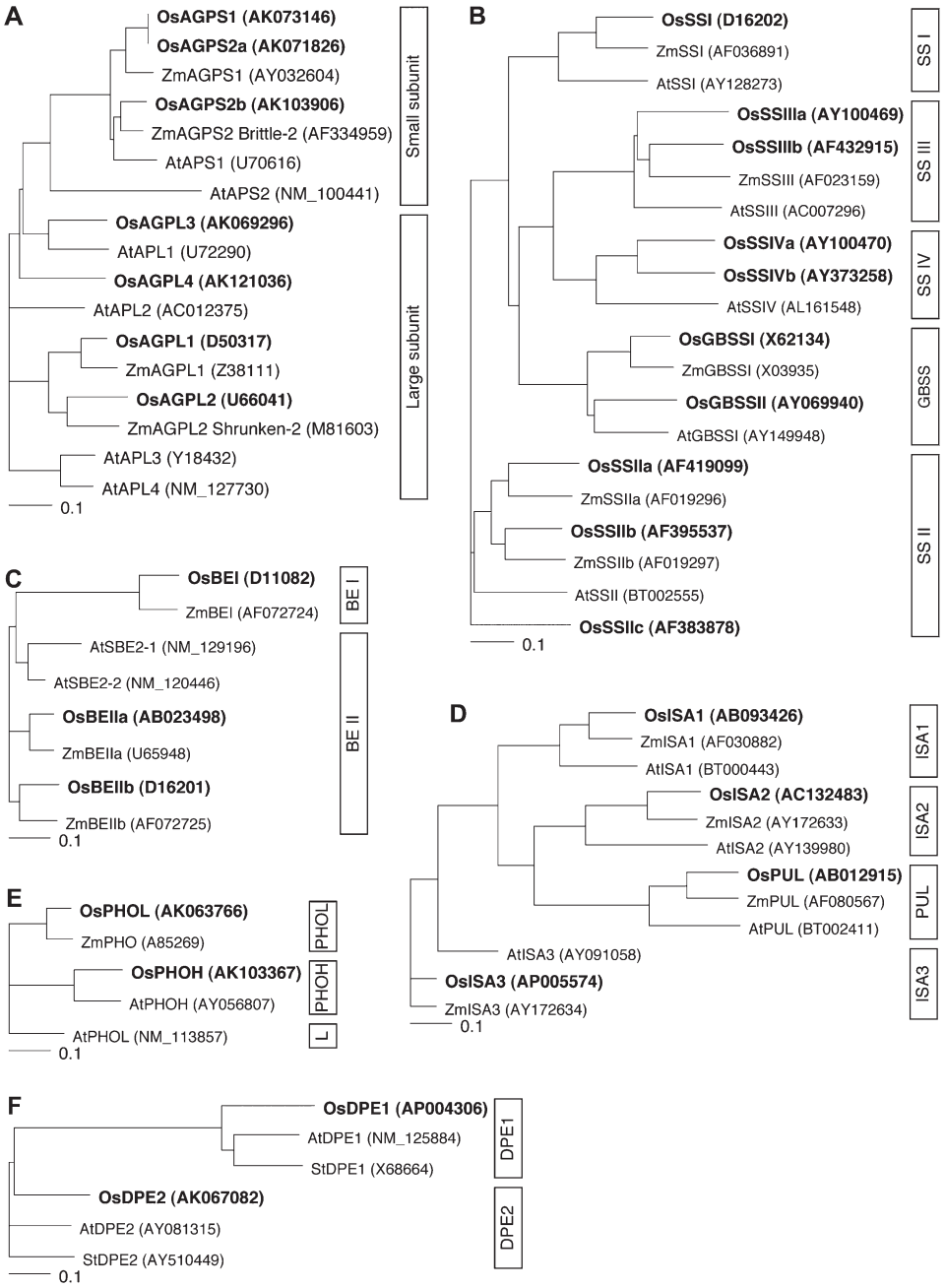

Fig. 1. Dendrograms of gene families encoding six classes of starch-synthesizing enzymes of rice (*Oryza sativa*), maize (*Zea mays*), *Arabidopsis thaliana*, and potato (*Solanum tuberosum*). Multiple sequence alignment analysis, using the CLUSTAL W program, was carried out for the deduced amino acid sequences of each enzyme class. (A) ADPglucose pyrophosphorylase (AGPase); (B) starch synthase [soluble starch synthase (SS) and granule-bound starch synthase (GBSS)]; (C) starch branching enzyme (BE); (D) starch debranching enzyme (DBE) [isoamylase (ISA) and pullulanse (PUL)]; (E) phosphorylase (PHO); and (F) disproportionating enzyme (DPE). The GenBank/KOME accession number of each gene is shown in parenthesis. Genes from rice, maize**,** *Arabidopsis*, and potato are indicated by the prefixes Os, Zm, At, and St, respectively. AGPL, AGPase large subunit; AGPS, AGPase small subunit.

(Fig. 1B). The *SSII* subclass appeared to be the most ancient. *SSIIc*, which had a homologue in *Arabidopsis* but not in maize, emerged much earlier than *SSIIa* and *SSIIb*. The rest of the *SS* genes arose from a common lineage from which *SSI* emerged first. Later coming out of the line were ones for the *SSIII* and *SSIV* genes and another for the *GBSS* genes. All *SS* genes had homologues in maize except for *SSIIc*, *SSIIIa*, *SSIVa*, and *SSIVb*, and *GBSSII* which had counterparts in *Arabidopsis*. All rice *BE* genes had corresponding genes in maize. *OsBEI* was likely to be more recent than *OsBEIIa* and *OsBEIIb* (Fig. 1C). Among the rice debranching enzymes, ISA3 may have evolved earliest,

followed by ISA1, ISA2, and then PUL (Fig. 1D). All these DBEs had their homologues in both maize and *Arabidopsis*. OsPHOL, the more ancient PHO, had a homologue in maize, and OsPHOH in *Arabidopsis* (Fig. 1E). Similarly, maize has no known counterpart to both the rice *DPE* genes, but *Arabidopsis* and potato did (Fig. 1F). However, because the maize genome has not yet been fully determined, it is probable that maize, also a monocot, has a counterpart for all the rice genes involved in starch synthesis. If these maize genes are finally identified, it is predicted that they will be proven to be phylogenetically closer to their homologues in rice than in *Arabidopsis*.

Description of the gene classes

AGPase: Plant AGPase is a heterotetrameric enzyme composed of two each of the larger regulatory subunits (AGPL) and the smaller catalytic subunits (AGPS) (Smith-White and Preiss, 1992; Preiss and Sivak, 1996; Greene and Hannah, 1998). Cereals have both plastidial and cytosolic AGPases (Denyer *et al*., 1996; Beckles *et al*., 2001; Hannah *et al*., 2001). Rice has a total of six genes encoding AGPase: two for AGPS (*OsAGPS1* and *OsAGPS2*) and four for AGPL (*OsAGPL1*, *OsAGPL2*, *OsAGPL3*, and *OsAGPL4*). The *AGPS2* gene apparently encodes the transcripts for *AGPS2a* and *AGPS2b*, which differ only in their first exons (*AGPS2a* exon 1 is encoded by a portion of the *AGPS2* gene that serves as intron 1 of *AGPS2b*), and are either processed from the common pre-mRNA by alternative splicing mechanisms or produced by different promoters. *AGPS2a* is a 1930 bp transcript and carries a putative transit peptide sequence, thus probably plastidial in location, while *AGPS2b* is 1749 bp and has no transit peptide making it probably a cytosolic form. Similarly, the barley AGPase highly homologous small subunit transcripts *bepsF1* (expressed in endosperm) and *blps14* (expressed in leaves) are transcripts of a single gene for the AGPase small subunit (Thorbjønsen *et al*., 1996*b*), and the wheat small subunit transcripts *AGP.S.1a* (260 bp, probably cytosolic) and *AGP.S.1b* (353 bp, probably plastidial) are derived from the *T.a.AGP.S.1* gene (Burton *et al*., 2002). In maize, however, the transcripts for the cytosolic and plastidial small subunits of AGPase were verified as products of two different genes (Hannah *et al*., 2001).

Judging from the presence of putative transit peptides and sequence homologies with corresponding AGPases from other species for which localization has been characterized (data not shown), OsAGPS1, OsAGPS2a, OsAGPL1, OsAGPL3, and OsAGPL4 are considered to be localized in plastids, whereas OsAGPS2b and OsAGPL2, which correspond to a maize Brittle-2 protein and a Shrunken-2 protein, respectively, are present in the cytosol (Bhave *et al*., 1990; Hannah *et al*., 2001).

SS and GBSS: It is generally accepted that soluble starch synthase (SS) and starch granule-bound starch synthase (GBSS) are involved in amylopectin and amylose synthesis, respectively, although the possibility that GBSS plays a role in forming long chains of amylopectin cannot be ruled out (Delrue *et al*., 1992).

There are four types of SS: SSI, SSII, SSIII, and SSIV, while GBSS has two types: GBSSI and GBSSII. Rice has a total of eight genes for SS: a single gene for SSI (*OsSSI*), three genes for SSII (*OsSSIIa*, *OsSSIIb*, and *OsSSIIc*), two genes for SSIII (*OsSSIIIa* and *OsSSIIIb*), and two genes for SSIV (*OsSSIVa* and *OsSSIVb*) (Hirose and Terao, 2004). A single gene each for GBSSI (*OsGBSSI*) and GBSSII (*OsGBSSII*) are present in rice (Hirose and Terao, 2004).

BE: Plants have two BE types, BEI and BEII. Rice contains a single gene for BEI (*OsBEI*) and two genes for BEII (*OsBEIIa* and *OsBEIIb*). It has been reported that *OsBEIIb* is specifically expressed in the endosperm whereas both *BEI* and *BEIIa* are expressed in all tissues examined (Yamanouchi and Nakamura, 1992; Mizuno *et al*., 1993).

DBE: Plants have two types of DBE, ISA and PUL, which differ in properties such as substrate specificity (Nakamura, 1996). Rice has three *ISA* genes (Kubo *et al*., 2005) and a single *PUL* gene (Nakamura *et al*., 1996).

PHO: Plant PHO is composed of two types, plastidial PHOL and cytosolic PHOH (Shimomura *et al*., 1982; Steup, 1988). A single gene each for PHOH and PHOL are present in rice.

DPE: Plants have the plastidial type DPE1 and the cytosolic type DPE2 (Lu and Sharkey, 2004). Rice has a single gene for each.

Measurement of transcript levels by RT-PCR analysis

Expression profiles of these rice genes were determined by quantitative real-time PCR analysis. The oligonucleotide primer pairs used for quantitative measurement of the specific amounts of transcript for individual genes of interest are listed in Table 1. To discriminate the two transcript types of the *AGPS2* gene, i.e. the *AGPS2a* and *AGPS2b* transcripts, the primer pairs were designed from their variable first exons. To verify the specificity of each primer set, their amplification products were cloned and sequenced. Plasmid DNAs containing these genes were used to establish standard curves, and the amount of mRNA synthesized by each gene was normalized as copy number per nanogram of total RNA according to Bustin (2000) and Gachon *et al*. (2004).

Expression patterns of each class of genes during endosperm development

Rice grain development in this study was divided into the following stages: initiation stage corresponding to 1–3 DAF when starch is synthesized exclusively in the pericarp and seed dry weight is relatively constant; early developmental stage (3–5 DAF) when seed total dry matter starts to

increase and endosperm starch begins to accumulate; middle stage (5–10 DAF) when endosperm starch and grain dry weight rapidly increase; and late stage (10 DAF onward) when both endosperm starch and seed weight continue to increase, then maximum values are reached and they become constant. The expression profiles throughout seed development for each class of genes are described below.

AGPase: *AGPL1* and *AGPS1* were vigorously expressed at the early phase of grain development, their transcripts being abundant from the onset of seed development, sharply rose to peak at 3 or 5 DAF then declined abruptly thereafter. By contrast, those of the two cytosolic forms, *AGPS2b* and *AGPL2*, were low until 3 DAF, then increased dramatically to peak at 5 DAF (Fig. 2A). All throughout seed development, the transcript level of *AGPL3* was relatively low while *AGPL4* and *AGPS2a* were scarcely expressed. These results suggest that AGPS1 combines with AGPL1 to form the plastidial AGPase, which is probably important before or/and at the early stage of endosperm/embryo development and/or pericarp. On the other hand, AGPS2b and AGPL2 possibly associate to form a heterotetrameric cytosolic AGPase, which is markedly expressed after 5 DAF to play an important role in starch accumulation via AGPglucose synthesis in endosperm.

SS and GBSS: The expression profiles of the *SS* and *GBSS* genes were highly variable (Fig. 2B). The *SSI* transcript level was already high at the earliest phase of seed formation (1–3 DAF), slightly increased to peak at 5 DAF when starch synthesis in the endosperm begins, then remained almost constant through the late-milking stage of endosperm development (15 DAF), suggesting that SSI is important at all stages of seed development. This is consistent with the fact that SSI is the major SS form in cereal endosperm (Cao *et al*., 1999).

The transcripts of *SSIIa* and *SSIIIa* rapidly increased from low levels at the onset of seed development to peak at 5–7 DAF and then continued to be significantly high. It is interesting that, among the *SS* genes, *SSIIa* appeared to be the most vigorously expressed, more than *SSI* or *SSIIIa*, whereas SSI and SSIIIa are known to account for the major SS activities in cereal endosperm (Cao *et al*., 1999).

By contrast, transcripts for *SSIIIb* were abundant at the start of grain formation until the early stage of seed development, but dropped precipitously at the onset of endosperm starch synthesis. On the other hand, *SSIVb* transcripts were low at the start then rose briefly at 5 DAF to the maximum level attained by *SSIIIb* and gradually decreased. Across all sampling periods, the transcripts of *SSIIIb* and *SSIVb* were consistently lower than those of *SSIIa*, *SSIIIa*, and *SSI*. The rest of the *SS* genes, i.e. *SSIIb*, *SSIIc*, and *SSIVa* were barely expressed in the seed, all having their maximum transcripts at the earliest period of grain formation and decreasing to basal levels at 5 DAF, the start of starch accumulation in the endosperm (Fig. 2B2).

While the *GBSSI* transcripts were low at the beginning of seed development, they dramatically increased at 5 DAF and remained abundant until the end of endosperm development (Fig. 2B1). Clearly, the amount of *GBSSI* transcript was markedly higher than *GBSSII* or any of the *SS* genes, its maximum level being more than 3-fold higher than that of the most vigorously expressed *SS* gene (*SSIIa*) and approximately 55-fold of the least-expressed *SS* gene (*SSIVa*). By stark contrast, the expression of *GBSSII* was very low, and limited only to the early stage of seed development (Fig. 2B2).

BE: Compared with the number of transcripts of the other two *BE*s, those of *BEIIa* were at least one order higher at the onset of the grain-development stage (1–3 DAF), as shown in Fig. 2C. This relatively higher transcript level, however, was transient because it started to dwindle at 5 DAF when endosperm starch started to accumulate. By contrast, the relatively low transcripts of *BEIIb* and *BEI* rapidly increased from 3 DAF to peak at 5 and 7 DAF, respectively, and despite some fluctuations remained high until 15 DAF. While the expression profiles of *BEIIb* and *BEI* were similar, *BEIIb* transcripts from 7–20 DAF were only about half of those for *BEI.* These patterns of changes in the transcript levels of the *BEII* isoforms are consistent with the findings of Mizuno *et al*. (2001). These observations might suggest that BEIIb and BEI are needed in great amounts during endosperm starch synthesis, particularly straight after the initiation process, whereas BEIIa plays an important role in processes occurring before efficient starch synthesis in the endosperm. As with other genes, however, the transcript levels of *BEII* isoforms are not necessarily the measures of their enzymatic activities. Illustrative of this reality in rice are the observations that, while the transcript level of *BEIIa* was much lower than that of *BEIIb* after 7 DAF (Fig. 2C), the activities of BEIIa and BEIIb are actually maintained at the same levels throughout endosperm development (data not shown).

It is interesting that *BEI* and *BEIIb* are expressed early in and throughout endosperm development in rice (Mizuno *et al*., 1993), maize (Gao *et al*., 1996), and sorghum (Mutisya *et al.*, 2003), whereas in wheat (Morell *et al*., 1997) and barley (Sun *et al*., 1998; Mutisya *et al*., 2003) the *BEI* gene is not expressed until relatively late in endosperm development as compared with the *BEIIb* gene.

DBE: All the *DBE*s had comparatively low transcript levels at the onset of seed development (1–3 DAF), which were maintained by *ISA2* and *ISA3* all throughout seed development. By contrast, the transcripts of *ISA1* and *PUL* rose steeply from 3 DAF to reach peak levels at 7 DAF of about 12- and 56-fold higher, respectively, then gradually decreased to lower but still significant levels at 20 DAF (Fig. 2D), suggesting that ISA1 and PUL play important roles in the whole endosperm starch accumulation.

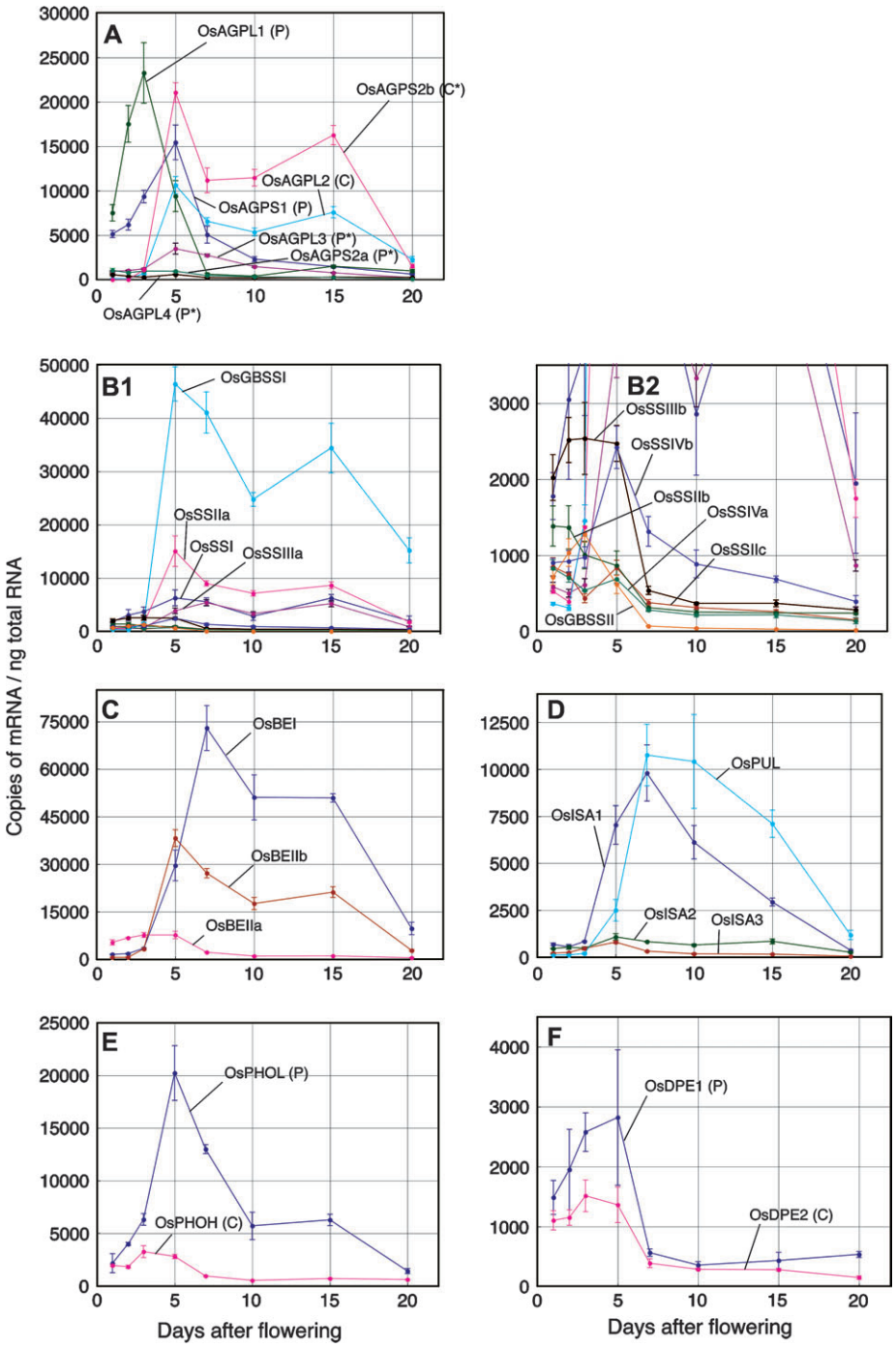

Fig. 2. Expression profiles of the six gene classes coding for starch-metabolizing enzymes during development of rice seeds. Rice spikelets were harvested at 1, 2, 3, 5, 7, 10, 15, and 20 d after flowering. An aliquot of the first strand of cDNA mixture corresponding to 5 ng of total RNA was used as template for quantitative real-time RT-PCR. Each value reported is the mean ±standard deviation of at least four independent measurements. P, Plastidic enzyme; C, cytosolic enzyme; P*, predicted plastidic AGPase; C*, predicted cytosolic AGPase.

PHO: *PHOL* transcripts were numerous at the onset of seed development, rapidly increased to peak at 5 DAF, and diminished to a low but significant level until 15 DAF, suggesting that PHOL plays an important part in starch biosynthesis throughout endosperm development. The transcripts of *PHOH*, which never exceeded those of *PHOL* were scarce from the start and dwindled from 5 DAF (Fig. 2E).

DPE: Although the absolute number of transcripts of *DPE2* was consistently lower than that for *DPE1*, the expression patterns of the two genes were similar (Fig. 2F). Their transcripts were already plentiful at the onset of seed

development, further increased to peak by 5 DAF, then abruptly decreased, suggesting a possibility that the involvement of both DPEs in seed development is confined to processes occurring before and/or at the initial stage of starch biosynthesis in rice endosperm.

Comparison of the expression patterns of each class of genes between leaf and endosperm

The expression patterns of the genes involved in starch metabolism were similar among young, fully developed, and old leaves, although in general young leaves had more transcripts than older ones (Fig. 3A). Because this trend across leaf age was apparent for the members of each gene class, only the data for the young leaf are reported.

AGPase: In young leaf, the transcript level of *AGPS2a* was about 11-fold higher than that of *AGPS1* (Fig. 3A) but the *AGPS2b* transcript was not detected (Fig. 3A). In seed, the transcript level for *AGPS2a* was consistently low while that for *AGPS2b*, although lower than *AGPS1* at the initial developmental stage, became higher at stages after 7 DAF (Fig. 2A). These data suggest that AGPS2a and AGPS2b play important roles in the synthesis of leaf transitory starch and endosperm storage starch, respectively. In green leaves, *AGPL3* transcripts were predominant, those of *AGPL1*, *AGPL2,* and *AGPL4* being scarce (Fig. 3A), while

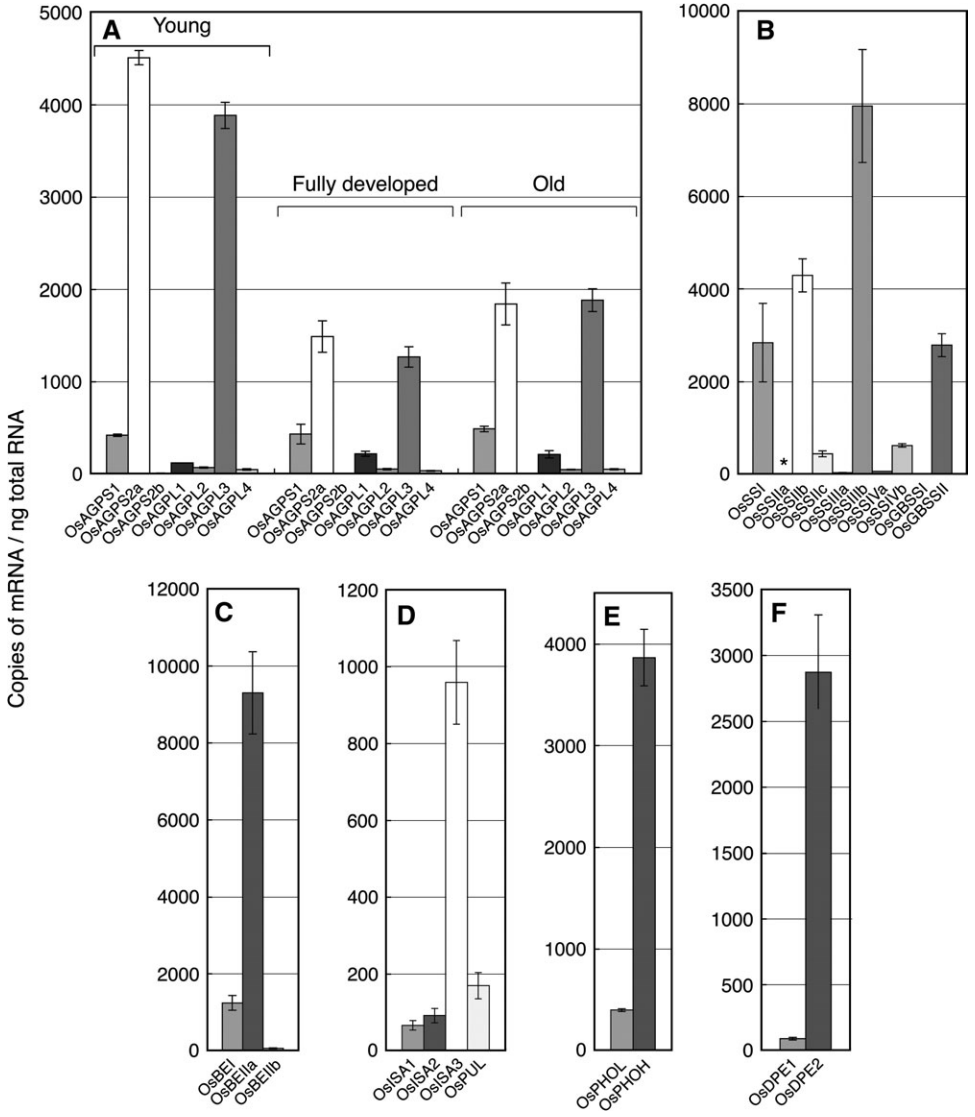

Fig. 3. Expression profiles of genes coding for the six classes of starch-metabolizing enzymes in rice leaf blade. (A) AGPase; (B) SS; (C) BE; (D) DBE; (E) PHO; and (F) DPE. Total RNAs were extracted from three leaf blades of 1-month-old plants: the uppermost expanding leaf (young); and the two below, the upper one considered as a fully developed leaf while the lower one as an old leaf. Aliquots of the first strand of cDNA mixture corresponding to 5 ng of the total RNA were used as template for quantitative real-time RT-PCR. Data from the fully developed and old leaves were provided only for *AGPase* genes (A). Each value is the mean ±standard deviation of at least four independent measurements. The asterisk indicates an extremely low and barely detectable transcript level.

in developing seed, *AGPL4* transcripts were very low as compared with those of the other three *AGPL* genes (Fig. 2A).

SS and GBSS: *SSI* was markedly expressed in developing endosperm (Fig. 2B) as well as in the leaf (Fig. 3B). Among the *SSII* genes, *SSIIb* was the major form expressed in the leaf while *SSIIa*, which was markedly expressed in the endosperm was scarcely expressed. The *SSIIc* transcript level was very low in both leaf and seed (Fig. 3B). *SSIIIb* was the only *SSIII* isoform expressed in the leaf (Fig. 3B) but, in seed during vigorous starch biosynthesis, *SSIIIa* expression was higher than *SSIIIb* (Fig. 2B). The transcript levels of *SSIVb* were higher than *SSIVa* in both leaf and endosperm (Figs 2B, 3B). The expression of *GBSSII* was leaf specific while *GBSSI* was endosperm specific (Figs 2B, 3B). These different expression patterns of *SS* and *GBSS* genes are consistent with previous reports (Hirose and Terao, 2004; Dian *et al*., 2005).

BE: The major *BE* forms expressed in the leaf were *BEIIa* and *BEI* (Fig. 3C), while *BEI* and *BEIIb* were predominant in the endosperm at the middle and late developmental stages (Fig. 2C). *BEIIb* expression was specific to endosperm, its transcript being very low in the leaf (Figs 2C, 3C). These results are in agreement with previous studies (Yamanouchi and Nakamura, 1992; Mizuno *et al*., 1993).

DBE: *ISA1* and *ISA2* were expressed only slightly in leaves but significantly in seeds (Figs 2D, 3D). *ISA3* was expressed markedly in the leaf but scarcely in the endosperm (Figs 2D, 3D). The *PUL* transcript level was significantly lower in the leaf than in the seed (Figs 2D, 3D).

PHO: *PHOH* expression was about 10-fold higher than *PHOL* in the leaf whereas in the endosperm, *PHOL* transcripts were about 6-fold higher than *PHOH* (Figs 2E, 3E).

DPE: *DPE2* was the predominant form in the leaf, whereas both *DPE1* and *DPE2* were significantly expressed in the endosperm but they were almost restricted to the early developmental stage (Figs 2F, 3F).

Effects of shrunken *mutation on expression of* AGPase *genes*

Results of recent studies indicate that the defective *AGPS2* in the *shrunken* mutant reduces the total AGPase activity to about 20% of the wild type (Kawagoe *et al*., 2005). Yano *et al*. (1984) reported previously that the AGPS2 protein is almost completely lacking in the endosperm, and that reduced AGPase activity causes a decline in starch accumulation in the endosperm, leading to shrivelling of the seed at maturity.

The transcripts of the cytosolic *AGPS2b*, which were absent from the leaves of the wild type and its *shrunken2* mutant EM22, were practically non-existent in the seeds of the mutant (Fig. 4). *AGPS2a* transcript abundance was comparable in the leaves of the mutant and the wild type but was more than 4-fold higher in the seeds of the mutant (Fig. 4). These results strongly suggest that the *shrunken* seed phenotype of EM22 is caused by the absence of transcripts for *AGPS2b*, probably the result of either mutation in the *AGPS2* gene itself or anomalies in the splicing of the pre-mRNA transcript. It is striking to note that the expressions of *AGPS1*, *AGPL1*, *AGPL3*, and especially *AGPL2* as well as *AGPS2a*, were dramatically enhanced in the seed (Fig. 4A). On the other hand, the most dramatic effect of the *shrunken* mutation in the leaf was the remarkable increase in transcript levels of both *AGPS1* and *AGPL1*, while that of *AGPL3*, the major AGPL isoform in the leaf, was unaffected by the mutation (Fig. 4B). These results indicate that the reduction of *AGPS2b* expression caused by the *shrunken* mutation greatly elevates the expression of the *AGPL* and other *AGPS* genes in the seed and leaf.

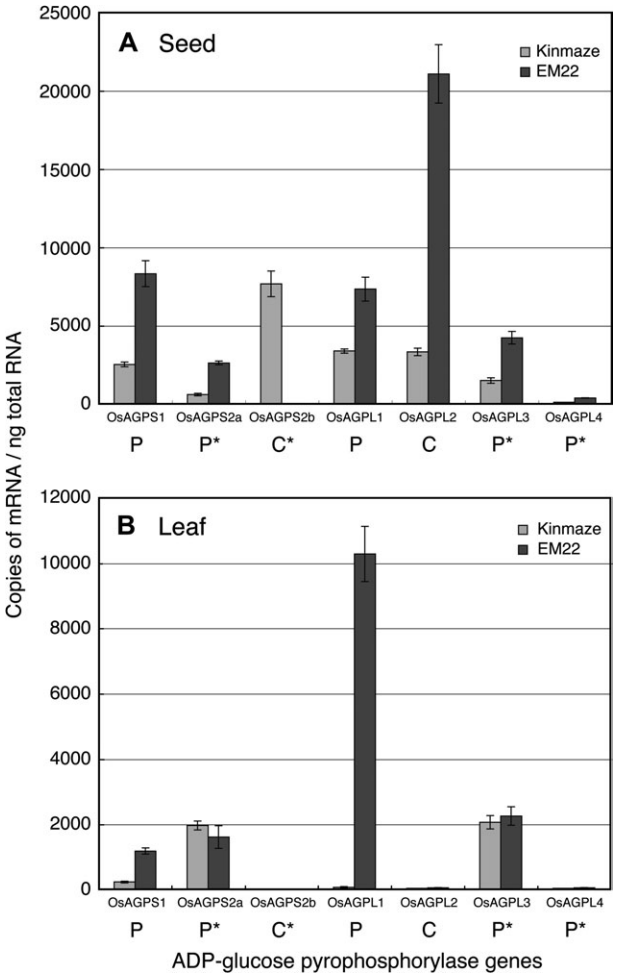

Fig. 4. Expression profiles of rice AGPase genes in developing endosperm and leaves of the *OsAGPS2*-deficient *shrunken* mutant line EM22 and its parent cultivar Kinmaze. An aliquot of the first strand of cDNA mixture corresponding to 5 ng of the total RNA was used as a template for quantitative real-time RT-PCR. Each value is the mean ±standard deviation of at least four independent measurements. (A) Seed; (B) leaf. P, Plastidial AGPase; C, cytosolic AGPase; P*, predicted plastidial AGPase; C*, predicted cytosolic AGPase.

Discussion

Amylopectin structure is influenced by starch-synthesizing enzymes

The fine structures as well as the relative amounts of amylopectin and amylose are specific for plant species and plant tissues where they are synthesized, and these features influence the physicochemical properties of starch granules. Several enzymes influence the fine structure of amylopectin, mainly by altering its cluster structure, although the modes of changes in amylopectin cluster structure strongly depend on the individual enzyme affected. In rice endosperm, several lines of evidence for this are available. First, SSIIa is responsible for the elongation of short-chains with DP5-10 to form longer A and B1 chains with DP11-24 within the cluster of amylopectin, and hence the difference in capacity for SSIIa discriminates the structural alteration of amylopectin between *indica*-type and *japonica*-type rice varieties having active SSIIa and inactive SSIIa, respectively (Umemoto *et al*., 2002; Nakamura *et al*., 2005). Secondly, BEIIb plays a specific role in the formation of A-chains of the amylopectin cluster (Nishi *et al*., 2001) and the levels of BEIIb activity affect the extent of structural changes in the cluster (Tanaka *et al*., 2004). Thirdly, ISA1 is essential for the formation of the highly organized cluster structure of amylopectin (Kubo *et al*., 1999) and the level of *ISA1* expression influences both the fine structure of amylopectin and starch thermal properties (Fujita *et al*., 2003; Kubo *et al*., 2005). These observations indicate that, in rice, the expression levels of sets of genes are programmed depending on the tissues and their developmental stages, otherwise each plant tissue cannot have a fixed structure of amylopectin, which is basically maintained throughout the life cycle. The tissue- and developmental stage-specific expression patterns of all genes for starch biosynthesis determine the fine structure of amylopectin in a particular tissue at a particular developmental stage, and hence the starch physicochemical characteristics, which influence the physiological status of cells. The physiological significance of the morphological and physicochemical properties of starch granules, however, is poorly understood.

The composition and the relative activities of isozymes of each class of the starch-synthesizing enzymes are known to differ between monocots and dicots. In most cereals (monocot), for instance, the BE class has several isozymes. In rice, the expression of the *BEIIb* gene is endosperm-specific while the *BEIIa* gene is ubiquitous (Yamanouchi and Nakamura, 1992; Mizuno *et al*., 1993, 2001). By contrast, dicots have either only a single *BEII*-type gene or multiple *BEII* genes, if any, that belong to the same gene subfamily (Sun *et al*., 1998). These differences in the relative activities and site of expressions of BE isoforms give rise to different X-ray diffraction patterns of starch granules, i.e. A-type in cereal grains, B-type or C-type in dicot organs (Hizukuri, 1996), and B-type in BEIIb-suppressed rice endosperm (Tanaka *et al*., 2004).

Expression profiling separates the genes involved in endosperm starch synthesis into four groups

The rice genome has a total of 27 candidate genes encoding the starch biosynthesizing enzymes and these are distributed in six classes as follows: six for AGPase, 10 for SS and GBSS, three for BE, four for DBE, two for PHO, and two for DPE. The present study analysed the transcriptome of these genes expressed in rice leaves and seeds as representative photosynthetic and non-photosynthetic organs, respectively. Detailed transcriptome analysis during seed endosperm development was also examined.

Results of the present study revealed four major patterns of expression of the rice starch-synthesizing genes during seed development, separating the enzymes into four groups (Fig. 2, Table 2; Supplemental Fig. 1). The first group is characterized by a high level of expression at a very early stage (initiation) of seed development, reaching a peak at 3 or 5 DAF followed by an abrupt decline, as exhibited by *OsAGPS1*, *OsAGPL1*, *OsSSIIIb*, *OsBEIIa*, *OsPHOH*, *OsDPE1*, and *OsDPE2*.

The second group, composed of *OsAGPL3*, *OsSSI*, *OsSSIVb*, *OsISA2*, and *OsPHOL*, has a medium expression level at initiation of grain formation, rapidly rises to peak at 5 DAF, gradually declines through 10 DAF, then either rises slightly at 15 DAF (*OsSSI* and *OsISA2*), or continues to decline. Because preliminary results show that ISA2 plays an important role in amylopectin biosynthesis by forming a functional heteromeric debranching enzyme protein with OsISA1, as found in potato tubers (Hussain *et al*., 2003), *OsISA2* has been classified into this group, although its transcript level is low.

The third group is characterized by a basal or a very low level of expression at the initial stage of seed development followed by a rapid increase in transcripts from about 5 or 7 DAF, which is maintained at significantly high levels until seed maturation. Possessing this pattern are the genes for *OsAGPS2b*, *OsAGPL2*, *OsSSIIa*, *OsSSIIIa*, *OsGBSSI*, *OsBEI*, *OsBEIIb*, *OsISA1*, and *OsPUL*. Since both *OsSSIIIa* and *OsISA1* are also expressed to some extent during the initial stage of the seed development, these enzymes may function in amylopectin synthesis from the very early stage of endosperm development.

The fourth group is characterized by low transcript level at the start (1–5 DAF) and further decreases to a basal, barely detectable level throughout the rest of seed development as exhibited by *OsAGPS2a*, *OsAGPL4*, *OsSSIIb*, *OsSSIIc*, *OsSSIVa*, *OsGBSSII*, and *OsISA3*.

Altogether, the above observations show that the timing of gene expression greatly differs among gene classes and among members of the same class, consistent with a previous report of different patterns of changes in enzyme

Table 2. *Grouping of rice genes involved in starch biosynthesis in the seed according to their individual expression patterns during grain development*

Dehulled developing seeds (including the endosperm and the pericarp) were analysed.

Gene group	Expression level at grain developmental stages			
	Initiation[a]	Early[b]	Middle[c]	Late[d]
	(1–3 DAF)	(3–5 DAF)	(5–10 DAF)	(10–20 DAF)
Group 1 *OsAGPS1*, *OsAGPL1*, *OsSSIIIb*, *OsBEIIa*, *OsPHOH*, *OsDPE1*, *OsDPE2*	High level	Increases to maximum level at 3 to/or 5 DAF	Abruptly declines to low level through 7 DAF then stabilizes	Stable low level
Group 2 *OsAGPL3*, *OsSSI*, *OsSSIVb*, *OsISA2*, *OsPHOL*	Medium level	Rapidly increases to reach peak at 5 DAF	Gradually declines to low level at 10 DAF	Continues to decline or briefly rises at 15 DAF
Group 3 *OsAGPS2b*, *OsAGPL2*, *OsSSIIa*, *OsSSIIIa*, *OsGBSSI*, *OsBEI*, *OsBEIIb*, *OsISA1*, *OsPUL*	Low level	Rapidly increases, some attaining peak level at 5 DAF	Slightly decreases or some continue to rise to reach peak level at 7 DAF	Slightly increases or gradually declines
Group 4 *OsAGPS2a*, *OsAGPL4*, *OsSSIIb*, *OsSSIIc*, *OsSSIVa*, *OsGBSSII*, *OsISA3*	Low level	Low level	Decreases to basal level	Constantly at basal level

[a] Grain starch not detected, and no increase in grain dry weight.
[b] Grain dry weight increases from 3 DAF, grain starch accumulates from 5 DAF.
[c] Rapid increases in grain starch content and in grain dry weight.
[d] Continued rapid increases in grain starch content and in grain dry weight.

activities during rice endosperm development (Nakamura and Yuki, 1992). The apparent grouping of many of the genes into the four expression patterns suggests that the physiological significance of starch synthesis dramatically changes at around 5 DAF during seed development. This particular period seems to be the exact timing when the seed starts to accumulate a huge amount of starch in the endosperm. It is assumed that the enzymes exhibiting the first pattern (the first group) play crucial roles in starch biosynthesis for the construction of the fundamental cell machineries in the embryo, aleurone layer, pericarp, and endosperm until about 3 DAF. It is also possible that the first-group enzymes play important roles in the *de novo* synthesis of glucan primers and the initiation of starch granules in the endosperm. The group 1 genes, together with the early but scarcely expressed group 4 genes, may also be involved in the synthesis of transitory starch in the seed pericarp. Recent work on *SS* genes in rice grains (Hirose and Terao, 2004) showed that the transcripts of *SSIIb*, *SSIIIb*, and *GBSSII* are mainly localized in the pericarp. These *SS* genes were designated as 'early expressers' because their transcripts lasted only until 5 DAF when endosperm starch evidently starts to accumulate.

It is assumed that PHO and DPE, which are usually considered to be involved in the process of starch degradation, may also play a role in the initial processes of starch biosynthesis in seeds, although their functions in starch biosynthesis have not been proven in plants, except in *Chlamydomonas* (Colleoni *et al*., 1999; Critchley *et al*., 2001).

At about 7 DAF or later, the endosperm cells are specialized to produce starch from sucrose and are destined to store the starch granules until amyloplasts enlarge to fill the whole cell space. At this time, the high expression of the enzymes belonging to the second and third groups could be closely related to the capacity of endosperm to synthesize starch efficiently. In this sense, it is reasonable to assume that all the enzymes that play specific roles in starch production in the endosperm are included in this category (second and third groups), because rice mutants lacking these enzymes such as SSIIa (Umemoto *et al*., 2002), BEI (Satoh *et al*., 2003*b*), BEIIb (Nishi *et al*., 2001), and ISA1 (Kubo *et al*., 1999) exhibit modified starch phenotypes in the endosperm (Nakamura, 2002; Satoh *et al*., 2003*a*).

The expression of the starch-synthesizing genes radically differs between leaves and seeds

The patterns of gene expression in the leaf sharply contrast with those in the seed (compare Fig. 3 with Fig. 2), as indicated by previous data on the activities of enzymes

involved in carbohydrate metabolism in leaves and seeds of rice (Nakamura *et al.*, 1989). Some genes such as *OsAGPS2* (for the *AGPS2a* transcript), *OsAGPL3*, *OsSSIIb*, *OsSSIIIb*, *OsGBSSII*, *OsBEIIa*, *OsISA3*, *OsPHOH*, and *OsDPE2* are specifically or markedly expressed in the leaf and none or only slightly in the seed, while *OsAGPS2* (for the *AGPS2b* transcript), *OsAGPL1*, *OsAGPL2*, *OsSSIIa*, *OsSSIIIa*, *OsGBSSI*, *OsBEIIb*, and *OsISA1* are preferably expressed in the seed (Table 3; Supplemental Fig. 2). The expressions of *OsAGPS2a*, *OsSSIIb*, *OsSSIIIb*, *OsGBSSII*, and *OsDPE2* are considered leaf specific while those for *OsAGPS2b*, *OsSSIIa*, *OsGBSSI*, and *OsBEIIb* are regarded as seed specific because their transcripts are not detected in the other organ (Table 3; Supplemental Fig. 2). *OsSSI* is highly expressed in both organs. There is no correlation between the organ-specific gene expression patterns and the developmental stage-specific expression patterns in the endosperm, as can be gleaned from Tables 2, 3; Supplemental Figs 1, 2).

The mechanism for organ-specific gene expression might reflect differences in the pathway for signal transduction of gene expression between the leaf as a photosynthetic organ and the endosperm as a non-photosynthetic organ, because light and sugars such as sucrose might be important possible factors regulating gene expressions in the leaf and the endosperm, respectively. A set of enzymes is required for the synthesis of the tandem-cluster structure of amylopectin (an organized arrangement of amylopectin and amylose molecules in the starch granule), and for determining the shape and size of the starch granule, which is organ and species specific. The involvement of different sets of enzymes in starch biosynthesis in source and sink organs might result in the different molecular structures of amylopectin and starch granule morphology, in agreement with the well-known fact that starch structure differs between source and sink organs to meet the physiological significance of starch as temporarily stored assimilatory material in source organs and as reserve material in sink organs (Tomlinson *et al.*, 1997).

The present data are basically consistent with the previous studies on the expression patterns of these genes in the seed and/or the leaf of rice plants (Mizuno *et al.*, 2001; Hirose and Terao, 2004; Jiang *et al.*, 2004; Dian *et al.*, 2005; Suzuki *et al.*, 2005).

Table 3. *Specific and preferential tissue localization of expression of rice genes involved in starch biosynthesis*

Expression in the endosperm is defined as 'specific' (underlined) and 'preferred' when the transcript level is at least 100-fold and 10-fold, respectively, of that in the leaf. Corresponding threshold values in the leaf are less than 0.1-fold and between 0.1–0.25-fold, respectively, of that in the endosperm.

Tissue	Genes
Leaf	*OsAGPS2a, OsAGPL3, OsSSIIb, OsSSIIIb, OsGBSSII, OsBEIIa, OsISA3, OsPHOH, OsDPE2*
Endsperm	*OsAGPS2b, OsAGPL1, OsAGPL2, OsSSIIa, OsSSIIIa, OsGBSSI, OsBEIIb, OsISA1*

Subunits of AGPase may form homotetrameric or heterotetrameric enzymes

Plant AGPase is a heterotetrameric enzyme composed of two large and two small subunits and its heteromeric nature has been extensively examined (Smith-White and Preiss, 1992; Greene and Hannah, 1998). AGPase catalyses the first committed reaction for starch biosynthesis by supplying SS with ADPglucose in plant tissues (Preiss and Sivak, 1996). There is compelling evidence indicating that, in some cereals such as maize, barley, rice, and wheat, cytosolic AGPase accounts for the major AGPase activity in the endosperm (Denyer *et al.*, 1996; Thorbjørnsen *et al.*, 1996*a*; Beckles *et al.*, 2001; Hannah *et al.*, 2001; Sikka *et al.*, 2001; Johnson *et al.*, 2003; Tetlow *et al.*, 2003). It is assumed that in rice endosperm, OsAGPS2b and OsAGPL2 associate to form a tetrameric cytosolic AGPase, because both proteins are the only known cytosolic AGPase small and large subunits, and are apparently co-ordinately expressed during endosperm development (Fig. 2A), consistent with the fact that maize cytosolic AGPase is composed of Brittle2 protein (small subunit) and Shrunken2 protein (large subunit) (Greene and Hannah, 1998). On the other hand, the expression patterns of plastidic *OsAGP* genes in the seed suggest that OsAGPS1 associates with OsAGPL1 to constitute the plastidic AGPase that functions at the onset of seed developmental stage up to 5 DAF, beyond which OsAGPL3 possibly replaces OsAGPL1 in the endosperm (Fig. 2A). In leaves, only the *OsAGPS2a* and *OsAGPL3* transcripts are abundant, the *OsAGPS1* gene being slightly expressed, whereas the transcript levels of *OsAGPL1*, *OsAGPL2*, and *OsAGPL4* are very low, and the *OsAGPS2b* transcripts are absent (Fig. 3A). These results suggest that in rice leaves, the major plastidial AGPase is a heterotetramer composed of OsAGPS2a and OsAGPL3, the less probable minor form being an AGPS1-AGPL3 heterotetramer, and it is likely that a cytosolic AGPase does not exist.

Reduced AGPS2b *transcripts exert dramatic pleiotropic effects on the transcript levels of other* AGPase *subunit genes*

The defective *OsAGPS2* gene in the *shrunken* mutation causes the complete loss of the *OsAGPS2b* transcript (Fig. 4) and the OsAGPS2 protein in rice endosperm (data not shown), and a reduction of the total AGPase activity to about 20% of the wild type (Kawagoe *et al.*, 2005). The *shrunken* mutation dramatically enhances the expression of other *OsAGP* genes in both seeds and leaves (Fig. 4). The expression of *OsAGPL2*, possibly the counterpart of *OsAGPS2b* for the cytosolic AGPase in the wild-type cultivar, is remarkably increased in the seed but not in the leaf, consistent with the observation by Giroux *et al.* (1994)

in maize endosperm that the mutation of either the *AGPS2* (*Brittle2*) or the *AGPL2* (*Shrunken2*) gene causes a marked increase in the transcript level of the counterpart gene. One possible explanation might be that in the cytosol of the mutant endosperm, OsAGPL2 forms a homotetramer that may or may not be catalytically active. The *shrunken* mutation also elevates the transcript levels for *OsAGPS1*, *OsAGPS2a*, *OsAGPL1*, and *OsAGPL3*, suggesting that the mutation enhances the plastidial *AGPase* expression and hence activity, presumably to compensate for the absence of AGPS2b. This may be the reason why the residual activity of AGPase in the mutant is quite high (about 20% of the wild-type activity), and the mutant can store starch in endosperm to some extent (Yano *et al*., 1984; Kawagoe *et al*., 2005). It is also interesting to note that the expressions of *OsAGPS1* and *OsAGPL1* remarkably increase in the leaf of the mutant, although the physiological significance and mechanism of these elevated *AGPase* gene expressions to the leaf chloroplasts are unknown.

Conclusions

The present results collectively provide concrete evidence that the expressions of the numerous genes involved in starch metabolism are highly co-ordinated temporally and spatially in both source and sink tissues of rice plants, keeping distinct expression patterns depending on individual genes; and that the changes in the expressions of some genes such as *OsAGPS2* due to the *shrunken* mutation can greatly affect the patterns of gene expression of other members of both its subclass (AGPS) and the other subclass (AGPL). Since every class of starch-synthesizing enzymes has multiple isoforms or subunits and all these enzymes are usually labile, it is impossible or very difficult to quantify separately all of these enzymatic activities or protein amounts in plant tissues, especially in the presence of interfering enzymes such as hydrolytic enzymes like amylases. Transcriptome analysis has an advantage of quantifying the changes in transcript levels of these genes at different developmental stages in wild-type species or their mutants and in cultivars with a different genetic background. Therefore, expression profiling of genes could lay a foundation for the identification of genes involved in the regulation of starch metabolism and provide valuable insights into the mechanism of metabolic regulation of starch biosynthesis under various physiological conditions. Nevertheless, it should be kept in mind that the transcript levels are not always related to their enzymatic activities or protein levels, considering post-transcriptional and post-translational controls.

The starch-biosynthesizing system in higher plants demands the concerted expression of many genes at any particular developmental stage of a particular tissue. In addition to maintaining the activity levels of individual enzymes, several combinations of protein–protein interactions such as those among the wheat proteins for BEI, BEIIb, and PHO (Tetlow *et al*., 2004) might play an essential role in the metabolic regulation of starch biosynthesis. Since it is difficult to measure separately the protein level or activity of each enzyme in the enzyme complex, transcriptome analysis would be a more convenient tool to reveal the regulatory mechanism for starch synthesis that determines when and where multiple genes are co-expressed and what proteins may interact.

Supplementary material

Supplementary figures are available online at www.jxb.oxfordjournals.org.

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References

Ball SG, Morell MK. 2003. From bacterial glycogen to starch: understanding the biogenesis of the plant starch granule. *Annual Review of Plant Biology* **54,** 207–233.

Beckles DM, Smith AM, ap Rees T. 2001. A cytosolic ADP-glucose pyrophosphorylase is a feature of graminaceous endosperms, but not of other starch-storing organs. *Plant Physiology* **125,** 818–827.

Bhave MR, Lawrence S, Barton C, Hannah LC. 1990. Identification and molecular characterization of shrunken-2 cDNA clones of maize. *The Plant Cell* **2,** 581–588.

Burton RA, Johnson PE, Beckles DM, Fincher GB, Jenner HL, Naldrett MJ, Denyer K. 2002. Characterization of the genes encoding the cytosolic and plastidial forms of ADP-glucose pyrophosphorylase in wheat endosperm. *Plant Physiology* **130,** 1464–1475.

Bustin SA. 2000. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *Journal of Molecular Endocrinology* **25,** 169–193.

Cao H, Imparl-Radosevich J, Guan H, Keeling PL, James MG, Myers AM. 1999. Identification of the soluble starch synthase activities of maize endosperm. *Plant Physiology* **120,** 205–215.

Chang S, Puryear J, Cairney J. 1993. A simple and efficient method for isolation of RNA from pine trees. *Plant Molecular Biology Reporter* **11,** 113–116.

Colleoni C, Dauvillée D, Mouille G, *et al.* 1999. Genetic and biochemical evidence for the involvement of α-1,4 glucanotransferases in amylopectin synthesis. *Plant Physiology* **120,** 993–1003.

Critchley JH, Zeeman SC, Takaha T, Smith AM, Smith SM. 2001. A critical role for disproportionating enzyme in starch breakdown is revealed by a knock-out mutant in Arabidopsis. *The Plant Journal* **26,** 89–100.

Delrue B, Fontaine T, Routier F, Decq A, Wieruszeski JM, Van Den Koornhuyse N, Mddelein M-L, Fournet B, Ball S. 1992. Waxy *Chlamydomonas reinhardtii*: monocellular algal mutant defective in amylose biosynthesis and granule-bound starch synthase accumulate a structurally modified amylopectin. *Journal of Bacteriology* **174,** 3612–3620.

Denyer K, Dunlap F, Thorbjørnsen T, Keeling P, Smith AM. 1996. The major form of ADP-Glc pyrophosphorylase in maize endosperm is extra-plastidial. *Plant Physiology* **112,** 779–785.

Dian W, Jiang H, Wu P. 2005. Evolution and expression analysis of starch synthase III and IV in rice. *Journal of Experimental Botany* **56,** 623–632.

Fujita N, Kubo A, Suh D-S, Wong K-S, Jane J-L, Ozawa K, Takaiwa F, Inaba Y, Nakamura Y. 2003. Antisense inhibition of isoamylase alters the structure of amylopectin and the physicochemical properties of starch in rice endosperm. *Plant and Cell Physiology* **44,** 607–618.

Gachon C, Mingam A, Charrier B. 2004. Real-time PCR: what relevance to plant studies. *Journal of Experimental Botany* **55,** 1445–1454.

Gallant DJ, Bouchet B, Baldwin PM. 1997. Microscopy of starch: evidence of a new level of granule organization. *Carbohydrate Polymers* **32,** 177–191.

Gao M, Fisher DK, Kim K, Shannon JC, Guiltinan MJ. 1996. Evolutionary conservation and expression patterns of maize starch branching enzyme I and IIb genes suggests isoform specialization. *Plant Molecular Biology* **30,** 1223–1232.

Giroux MJ, Boyer C, Feix G, Hannah LC. 1994. Coordinated transcriptional regulation of storage product genes in the maize endosperm. *Plant Physiology* **106,** 713–722.

Greene TW, Hannah LC. 1998. Maize endosperm ADP-glucose pyrophosphorylase SHRUNKEN2 and BTITTLE2 subunit interactions. *The Plant Cell* **10,** 1295–1306.

Hannah LC, Shaw JR, Giroux MJ, Reyss A, Prioul JL, Bae JM, Lee JY. 2001. Maize genes encoding the small subunit of ADP-glucose pyrophosphorylase. *Plant Physiology* **127,** 173–183.

Hirose T, Terao T. 2004. A comprehensive expression analysis of the starch synthase gene family in rice (*Oryza sativa* L.). *Planta* **220,** 9–16.

Hizukuri S. 1996. Starch: analytical aspects. In: Eliasson AC, ed. *Carbohydrates in food.* New York: Marcel Dekker, 347–428.

Hussain H, Mant A, Seale R, *et al.* 2003. Three isoforms of isoamylase contribute different catalytic properties for the debranching of potato glucans. *The Plant Cell* **15,** 133–149.

James MG, Denyer K, Myers AM. 2003. Starch synthesis in the cereal endosperm. *Current Opinion in Plant Biology* **6,** 215–222.

Jiang H, Dian W, Liu F, Wu P. 2004. Molecular cloning and expression analysis of three genes encoding starch synthase II in rice. *Planta* **218,** 1062–1070.

Johnson PE, Patron NJ, Bottrill AR, Dinges JR, Fahy BF, Parker ML, Waite DN, Denyer K. 2003. A low-starch barley mutant, Risø, lacking the cytosolic small subunit of ADP-glucose pyrophosphorylase, reveals the importance of the cytosolic isoforms and the identity of the plastidial small subunit. *Plant Physiology* **131,** 684–696.

Kawagoe Y, Kubo A, Satoh H, Takaiwa F, Nakamura Y. 2005. Roles of isoamylase and ADP-glucose pyophosphorylase in starch granule synthesis in rice endosperm. *The Plant Journal* **42,** 164–174.

Kubo A, Fujita N, Harada K, Matsuda T, Satoh H, Nakamura Y. 1999. The starch-debranching enzyme isoamylase and pullulanase are both involved in amylopectin biosynthesis in rice endosperm. *Plant Physiology* **121,** 399–409.

Kubo A, Rahman S, Utsumi Y, *et al.* 2005. Complementation of *sugary-1* phenotype in rice endosperm with the wheat *Isoamylase1* gene supports a direct role for isoamylase1 in amylopectin biosynthesis. *Plant Physiology* **137,** 43–56.

Lu Y, Sharkey TD. 2004. The role of amylomaltase in maltose metabolism in the cytosol of photosynthetic cells. *Planta* **218,** 466–473.

Mizuno K, Kawasaki T, Shimada H, Satoh H, Kobayashi E, Okumura S, Arai Y, Baba T. 1993. Alteration of the structural properties of starch components by the lack of an isoform of starch branching enzyme in rice seeds. *Journal of Biological Chemistry* **268,** 19084–19091.

Mizuno K, Kobayashi E, Tachibana M, Kawasaki T, Fujimura T, Funane K, Kobayashi M, Baba T. 2001. Characterization of an isoform of rice starch branching enzyme, RBE4, in developing seeds. *Plant and Cell Physiology* **42,** 349–357.

Morell MK, Blennow A, Kosar-Hashemi B, Samuel MS. 1997. Differential expression and properties of starch branching enzyme isoforms in developing wheat endosperm. *Plant Physiology* **113,** 201–208.

Mutisya J, Sathish P, Sun C, Andersson L, Ahlandsberg S, Baguma Y, Palmqvist S, Odhiambo B, Åman P, Jansson C. 2003. Starch branching enzymes in sorghum (*Sorghum bicolor*) and barley (*Hordeum vulgare*): comparative analysis of enzyme structure and gene expression. *Journal of Plant Physiology* **160,** 921–930.

Nakamura Y. 1996. Some properties of starch debranching enzymes and their possible role in amylopectin biosynthesis. *Plant Science* **121,** 1–18.

Nakamura Y. 2002. Towards a better understanding of the metabolic system for amylopectin biosynthesis in plants: rice endosperm as a model tissue. *Plant and Cell Physiology* **43,** 718–725.

Nakamura Y, Francisco Jr PB, Hosaka Y, Sato A, Sawada T, Kubo A, Fujita N. 2005. Essential amino acids of starch synthase IIa differentiate amylopectin structure and starch quality between *japonica* and *indica* rice varieties. *Plant Molecular Biology* **58,** 213–227.

Nakamura Y, Umemoto T, Ogata N, Kuboki Y, Yano M, Sasaki T. 1996. Starch debranching enzyme (R-enzyme or pullulanase) from developing rice endosperm. *Planta* **199,** 209–218.

Nakamura Y, Yuki K. 1992. Changes in enzyme activities associated with carbohydrate metabolism during development of rice endosperm. *Plant Science* **82,** 15–20.

Nakamura Y, Yuki K, Park S, Ohya T. 1989. Carbohydrate metabolism in the developing endosperm of rice grains. *Plant and Cell Physiology* **30,** 833–839.

Nishi A, Nakamura Y, Tanaka N, Satoh H. 2001. Biochemical and genetic analysis of the effects of *amylose-extender* mutation in rice endosperm. *Plant Physiology* **127,** 459–472.

Okagaki RJ. 1992. Nucleotide sequence of a long cDNA from the rice waxy gene. *Plant Molecular Biology* **19,** 513–516.

Preiss J, Sivak M. 1996. Starch synthesis in sinks and sources. In: Zamski E, Schaffer AA, eds. *Photoassimilate distribution in plants and crops: source–sink relationships.* New York: Marcel Dekker, 139–168.

Satoh H, Nishi A, Fujita N, Kubo A, Nakamura Y, Kawasaki T, Okita TW. 2003*a*. Isolation and characterization of starch mutants in rice. *Journal of Applied Glycoscience* **50,** 225–230.

Satoh H, Nishi A, Yamashita K, Takemoto Y, Tanaka Y, Hosaka Y, Sakurai A, Fujita N, Nakamura Y. 2003*b*. Starch-branching enzyme I-deficient mutation specifically affects the structure and properties of starch in rice endosperm. *Plant Physiology* **133,** 1111–1121.

Shimomura S, Nagai M, Fukui T. 1982. Comparative glucan specificities of two types of spinach leaf phosphorylase. *Journal of Biochemistry* **91,** 703–717.

Sikka VK, Choi SB, Kavakli IH, Sakulsingharoj C, Gupta S, Ito H, Okita TW. 2001. Subcellular compartmentation and allosteric regulation of the rice endosperm ADPglucose pyrophosphorylase. *Plant Science* **161,** 461–468.

Smith SM, Fulton DC, Chia T, Thorneycroft D, Chapple A, Dunstan H, Hylton C, Zeeman SC, Smith AM. 2004. Diurnal changes in the transcriptome encoding enzymes of starch metabolism provide evidence for both transcriptional and posttranscriptional regulation of starch metabolism in Arabidopsis leaves. *Plant Physiology* **136,** 2687–2699.

Smith-White BJ, Preiss J. 1992. Comparison of proteins of ADP-glucose pyrophosphorylase from diverse sources. *Journal of Molecular Evolution* **34,** 449–464.

Steup M. 1988. Starch degradation. In: Preiss J, ed. *The biochemistry of plants*, Vol. 14. *Carbohydrates.* San Diego: Academic Press, 255–296.

Sun C, Sathish P, Ahlandsberg S, Jansson C. 1998. The two genes encoding starch-branching enzymes IIa and IIb are differentially expressed in barley. *Plant Physiology* **118,** 37–49.

Suzuki K, Hattori A, Tanaka S, Masumura T, Abe M, Kitamura S. 2005. High-coverage profiling analysis of genes expressed during rice seed development, using an improved amplified fragment length polymorphism technique. *Functional Integrative Genomics* **5,** 117–127.

Tanaka N, Fujita N, Nishi A, Satoh H, Hosaka Y, Ugaki M, Kawasaki S, Nakamura Y. 2004. The structure of starch can be manipulated by changing the expression levels of starch branching enzyme IIb in rice endosperm. *Plant Biotechnology Journal* **2,** 507–516.

Tetlow IJ, Davies EJ, Vardy KA, Bowsher CG, Burrell MM, Emes MJ. 2003. Subcellular localization of ADPglucose pyrophosphorylase in developing wheat endosperm and analysis of the properties of a plastidial isoform. *Journal of Experimental Botany* **54,** 715–725.

Tetlow IJ, Wait R, Lu Z, Akkasaeng R, Bowsher CG, Esposito S, Kosar-Hashemi B, Morell MK, Emes MJ. 2004. Protein phosphorylation in amyloplasts regulates starch branching enzyme activity and protein–protein interactions. *The Plant Cell* **16,** 694–708.

Thompson DB. 2000. On the non-random nature of amylopectin branching. *Carbohydrate Polymers* **43,** 223–239.

Thorbjørnsen T, Villard P, Denyer K, Olsen O, Smith AM. 1996*a*. Distinct isoforms of ADPglucose pyrophosphorylase occur inside and outside the amyloplast in barley endosperm. *The Plant Journal* **10,** 243–250.

Thorbjørnsen T, Villard P, Kleczkowski LA, Olsen OA. 1996*b*. A single gene encodes two different transcripts for the ADP-glucose pyrophosphorylase small subunit from barley (*Hordeum vulgare*). *Biochemical Journal* **313,** 149–154.

Tomlinson K, Lloyd JR, Smith AM. 1997. Importance of isoforms of starch-branching enzyme in determining the structure of starch in pea leaves. *The Plant Journal* **11,** 31–43.

Umemoto T, Yano M, Shomura A, Satoh H, Nakamura Y. 2002. Mapping of a gene responsible for the difference in amylopectin structure between japonica-type and indica-type rice varieties. *Theoretical and Applied Genetics* **104,** 1–8.

Yamanouchi H, Nakamura Y. 1992. Organ specificity of isoforms of starch branching enzyme (Q-enzyme) in rice. *Plant and Cell Physiology* **33,** 985–991.

Yano M, Isono Y, Satoh H, Omura T. 1984. Gene analysis of *sugary* and *shrunken* mutants of rice, *Oryza sativa* L. *Japanese Journal of Breeding* **34,** 43–49.