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Screening of EST Clones Influencing Amylose Synthesis in Wheat Endosperm using cDNA Microarray

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ABSTRACT. *Genetic factors influencing amylose synthesis in wheat endosperm were investigated by microarray technology. The array was developed by duplicates of 3,367 selected expressed sequence tags (ESTs) from two libraries of a wheat cultivar chinese spring (CS); one from developing grains at 10 days post anthesis (DPA) and another from 30 DPA. Differential expression of ESTs in three near-isogenic genotypes with different Wx gene composition, the wild type CS, a partial waxy type and the perfect waxy type, were analyzed using total RNA of immature grains at 10 DPA. Of the tested ESTs, about 1% (28 clones) showed differential expressions among genotypes and, according to the BLAST analysis, 18 of those displayed homologous counterparts. Expression of those clones was highest in CS, followed by the partial and the perfect waxy type. Most of the functionally known ESTs either belonged to chloroplast DNA or genes coding for ribosomal RNA. UDP-Glucose pyrophosphorylase, one of the identified clones involved in the starch synthesis pathway, was expressed highly in CS than in the other two waxy genotypes.*

INTRODUCTION

Cereal starches are made up of two glucose polymers, amylose and amylopectin. Starch structure, especially amylose to amylopectin ratio, is a crucial determinant for many end-uses of starch. Production of structurally modified natural starches has been the interest of recent research. Despite the fact that the starch is a combination of two monomeric polymers, there are many questions still to be resolved in term of its biosynthesis, regulations, deposition in granules and initiation and development of starch granules (Ball *et al.*, 1998; Smith, 1999).

Starch synthesis is started with the conversion of ADP-Glucose to amylose and amylopectin by two independent pathways. Amylose synthesis has been well studied and

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reviewed (Smith *et al.*, 1997; Denyer *et al.*, 2001; Zeeman *et al.*, 2002). The content of amylose is mainly determined by a major gene called the *Wx* gene, which encodes the Wx protein or granule-bound starch synthase I (GBSS I), the enzyme responsible for amylose biosynthesis in the endosperm (Martin and Smith, 1995). In hexaploid wheat (*Triticum aestivum* L.) the *Wx* genes are organized as a triplicate set of single-copy homoeoloci (Chao *et al.*, 1989). These three *Wx* genes have different effects on apparent amylose content and starch properties (Miura *et al.*, 1999; Araki *et al.*, 2000; Yamamori and Quynh, 2000; Wickramasinghe *et al.*, 2003). Other than the *Wx* gene, QTL (Araki *et al.*, 1999) and genes associated with amylopectin biosynthesis (Miura *et al.*, 1994; Yamamori *et al.*, 2000) have shown some effects on the final amylose content in wheat endosperm. In rice, some modifiers were also proven as the possible causes in changing the amylose content (Mckenzie and Rutger, 1983; Okuno *et al.*, 1983; Mikami *et al.*, 2000).

Biosynthesis of starch and protein storage products are initiated simultaneously during the second phase of seed development (6-13 days post anthesis) (Simmonds and O'Brien, 1981). Therefore, those anabolic reactions with complicated pathways comprising a large number of enzymatic reactions disturb the exhaustive identification of genes associated with amylose synthesis using classical genetic approaches. Recently, Ogihara *et al.* (2003) have produced a body map of ESTs in typical tissues during the wheat life cycle, and hence cDNA microarrays are available to monitor gene expression in the seed at the stage of starch synthesis. This encouraged us to find the Espressed Sequence Tags (ESTs) associated with amylose biosynthesis using the microarray technology. Thus, in the present experiment genetic factors influencing amylose synthesis in near-isogenic genotypes with different *Wx* gene composition were investigated by microarray technology.

MATERIALS AND METHODS

Plant materials

A near-isogenic population of doubled haploid lines were taken for the study that were developed by Miura *et al.* (2002) from F_1 plants of chinese spring (CS) x waxy CS through wheat x maize method. Of the possible eight types of lines with different *Wx* alleles, CS, waxy CS and wxBD were chosen as testing materials in the present experiment. CS is the wild type that bears wild type alleles at all three *Wx* loci, *Wx-A1*, *Wx-B1* and *Wx-D1*. The waxy CS possesses null alleles at all three *Wx* loci and wxBD is a double null type, which has the wild type alleles only at the locus of *Wx-A1*.

For RNA extraction, the three genotypes were grown in a growth chamber maintained at 20 °C during the day and 16 °C at night and a day-length of 16 hrs.

Isolation of sample RNA

Total RNA of these three genotypes was extracted from immature grains at 10 days post anthesis (DPA) using SDS-phenol method (Ozeki *et al.*, 1990). They were used for microarray hybridization, RT-PCR and northern hybridization.

Total RNA of each genotype was reverse-transcribed in the presence of Cy3-dUTP or Cy5-dUTP (Amersham Pharmacia Biotech, UK). Each reaction was carried out in a 20 µL volume containing 20 µg of total RNA, 1 µL of random hexamer (Roche, Germany), 500 µM dATP, dCTP, dGTP, 200 µM of dTTP, 100 µM Cy3-dUTP or Cy5-dUTP, 10 µM DTT, and 400 units of superscript II reverse transcriptase (Gibco, USA) in reaction buffer (Gibco, USA). After incubation at 42 °C for 60 min, the temperature of the reaction mixture was elevated to 94 °C and kept at this temperature for 3 min. Then 2 µL of 2.5 N NaOH was added to the solution, which was then incubated at 37 °C for 15 min. The samples were neutralized by adding 10 µL of 2 M MOPS, and purified with a QIA quick purification kit (QIAGEN K. K., Japan) according to the manufacturer's instructions. Labeled DNA was precipitated with ethanol, and dried under vacuum. The pellet was dissolved in 25 µL of hybridization buffer with 5 x SSC and 0.5% SDS and was used for microarray hybridization.

cDNA library and construction of microarray

Out of 10 cDNA libraries, 2 libraries constructed using poly (A+) RNA of immature seeds at 10 DPA and 30 DPA of CS at Kihara Institute for Biological Research, Yokohama, Japan, were used. After grouping and clustering ESTs (Ogihara *et al.*, 2003), about 3300 ESTs isolated from 10 and 30DPA seeds were selected for microarray preparation. Those selected ESTs together with positive and negative controls were printed in duplicate onto the microscope slides (Matsunami: S-0137, Japan) according to the design of the Kihara Institute for Biological Research, Yokohama, Japan and, coated with poly-L-lysine (Sigma, USA) by using a 16-pin printed microarrayer (SPBIO, Hitachi software Engineering Co. Ltd. Japan).

Array hybridization

Flourescence-incorporated cDNA solutions were boiled for 2 min and quickly applied to the microarray under a cover slip. The microarray was placed in the hybridization chamber and incubated for 16 hrs at 65 °C. After hybridization, the arrays were sequentially washed in the following solutions at room temperature: 2 x SSC with 0.1% SDS for 10 min, 0.2 x SSC with 0.1% SDS for 10 min, rinsed in 0.2 x SSC, and 0.05 x SSC. The arrays were dried by centrifugation at 1000 rpm for 10 sec (Himac CR-21F, Hitachi Co. Ltd. Japan), and then scanned with a scan Array 4000 (GSI Lumonics, Watertown, MA, USA). A separate scan was carried out for each of the two fluorescent dyes. In order to normalize the signal intensities of two series, the photomultiplier and the laser power were adjusted. Consequently, the signal ratio of most of the control genes between two data acquisitions was close to 1.0. Labeling of RNA and hybridization of arrays were performed at Kihara Institute for Biological Research, Yokohama, Japan.

Data analysis

The integrated optical density of each gene on the array for each probe was measured by using the software ArrayGauge (Fuji film Co. Ltd, Tokyo, Japan). The quantified data were analyzed using Microsoft Excel. The background intensity of each spot was deducted from the total intensity of the spot. The ratio between intensities at Cy5/Cy3

was calculated and this value was converted to $\log_2$. After normalization, the ESTs showed more than 2-fold differences between the intensity of both channels were selected.

Further, the intensity (total intensity-background intensity) of clones in each channel were arranged in descending order and clones which showed higher intensity (the first 35 clones, about 1%) were searched for their homologies in Komugi integrated wheat science data base (http://www.grs.nig.ac.jp/wheat/komugi/).

RT-PCR

In order to confirm microarray analysis, RT-PCR was preformed with the selected clones. Specific primer pairs for each clone were developed by the software Primer3. RT-PCR was carried out according to the manufacturer's instructions with modifications in PCR conditions [RNA PCR kit (AMV) var. 2.1, Takara Bio Inc, Japan].

Northern hybridization

Northern hybridization was done according to Kohri *et al.* (1993). Total RNA (5 µg) was denatured at 65°C for 10 min and electrophoresed on 1% denaturing agarose gel in 1 x MOPS buffer. Fractionated RNAs were transferred to nylon membranes (45 µ Pall Biodyne B membrane, Nippon Genetics Co Ltd, Japan). Selected ESTs from the microarray analysis were labeled and used as probes. The probes were labeled by DIG labeling method as manufacturer's instructions (DIG RNA labeling mix, Roche Diagnostics GmbH, Germany).

RESULTS

Microarry

3367 ESTs were spotted on each array in duplicates. The experiment was designed with two combinations as CS/wxBD and wxBD/waxy CS. Each combination had two arrays. After normalization, the clones which showed highest intensity at each channel were separately observed. They could be divided into three groups as genes coding for storage proteins subunits, rRNA (18S and 26S) and, unknown clones (Table 1).

In differential expression analysis, 28 ESTs showed more than 2-fold differences at the intensities of two channels, Cy3 and Cy5. Out of them, 6 clones showed differential expression in both test combinations, 3 clones only in wxBD/waxy CS and 19 in CS/wxBD. The expressions of all ESTs were highest in CS followed by wxBD and waxy CS.

The sequence data of those 28 clones were retrieved from "Komugi" Integrated Wheat Science database and are listed in Table 2. Ten clones were unknown while 18 showed homology to other ESTs or clones with known functions. Most of them were genes or part of the genes related to rRNA synthesis (11 ESTs), or were found in ctDNA (2 ESTs).

Table 1. The most abundantly expressed clones of the three near-isogenic waxy lines at 10 DPA

Clone	Definition
Whsl31l23	emb|X03346.1|TAGLUT1 Wheat gene for HMW glutenin subunit
Whe15o23	emb|X03346.1|TAGLUT1 Wheat gene for HMW glutenin subunit
Whe12d08	emb|X03346.1|TAGLUT1 Wheat gene for HMW glutenin subunit
Whe20e12	dbj|AB042240.3| *T. aestivum* ct DNA, complete genome
Whe14o11	Unknown
Whe15j08	emb|AJ130948.1|TSP130948 *T. spelta* alpha-gliadin gene, partial
Whe17e19	gb|AY049041.1| *T. aestivum* 28S rRNA gene, partial sequence
Whe20e02	gb|AF280605.1|AF280605 *T. aestivum* omega gliadin storage protein gene, complete cds
Whe20e18	Unknown
Whsl10p11	emb|X03346.1|TAGLUT1 Wheat gene for HMW glutenin subunit
Whe25l14	emb|X13927.2|TAGLB11B *T. aestivum Glu-B1-1b* gene for HMW glutenin subunit
Whsl21f18	emb|X03346.1|TAGLUT1 Wheat gene for HMW glutenin subunit
Whe16g02	Unknown
Whe12m03	Unknown
Whe20o22	gb|J01309.1|WHTGLG Wheat storage protein (gamma-gliadin) mRNA, partial

The remaining five ESTs which had homology to functional genes were expressed differentially only in the test combination of CS/wxBD and not in the combination of wxBD/waxy CS. Out of them, two would be directly related to starch synthesis [genes encode for 26S proteasome regulatory particle triple-A ATPase subunit 4 and UDP-glucose phyrophosphorylase (UDPase)]. The others showed homology to Ty1-copia retrotransposon OARE-1 RNH pseudogene for RNaseH of wheat, eukaryotic initiation factor-5 gene exon1-2 of maize and, actin-1 gene of *T. monococcum*.

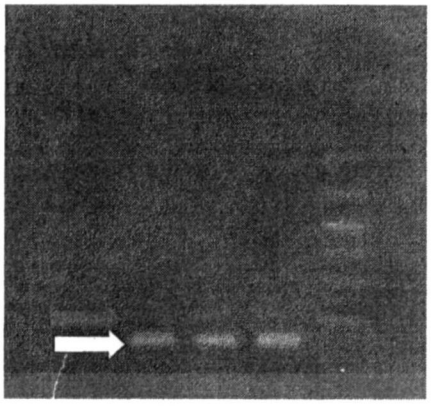
Table 2. Differentially expressed 28 clones among the three near-isogenic waxy lines

Clone	BLAST (count)	Expression ratio	Remarks
Whe11p20	27	9. 9.1-11.9	*T. aestivum* ct DNA complete se.
Whe14d20	500	2.2-2.9	*T. aestivum* 18S rRNA gene, partial se.
Whe14l17	41	2.9-3.1	*Secale cereale* chloroplast *psbD*, *psbC* and *trnS* genes for D2 polypeptide, Chl a-binding protein and tRNA-Ser (uga), respectively
Whe14o11		2.0	Unknown
Whe20k12	8	2.1-2.4	*T. aestivum* Ty1-copia retrotransposon OARE-1 RNH pseudogene for RNase H
Whe20g11		2.0	Unknown
Whe21d16		2.4	Unknown
Whe23j06	500	2.8-6.7	*T. aestivum* ct DNA, complete se.
Whe25c01	500	2.5-3.0	*T. aestivum* 18S rRNA gene, partial se.
Whe6e21	2	2.2	*T. monococcum* actin (ACT-1) gene, partial se.; chromosome condensation factor (CCF), resistance protein (RGA-2), and nodulin-like protein (NLL) genes, complete se.; and retrotransposon
Whe7l10		2.8	Unknown
Whsl14h22		2.1-2.3	Unknown
Whsl14j21	2	2.1-2.2	*O. sativa* OsRPT4 mRNA for 26S proteasome regulatory particle triple-A ATPase subunit 4
Whsl1p03	500	2.1	*Secale cereale* 26S rRNA 3' end and 18S rRNA, 5' end
Whsl1k21	500	2.7-3.1	*T. aestivum* 18S rRNA gene, partial se.
Whsl18b13	8	2.8	*T. aestivum* 18S rRNA gene, partial se.
Whsl24j01	500	2.2-3.0	*T. aestivum* 18S rRNA gene, partial se.
Whsl24m07	28	2.2-3.6	*T. spelta* 26S rRNA and 18S rRNA genes, partial se.; and intergenic region
Whsl25j24	500	2.4-3.5	*T. aestivum* 18S rRNA gene, partial se.
Whsl27d06		2.1-2.5	Unknown
Whsl29i05	500	2.1-3.2	*T. aestivum* 18S rRNA gene, partial se.
Whsl31d07	500	2.7	*T. aestivum* 18S rRNA gene, partial se.
Whsl5g10	17	2.4	*H. vulgare* mRNA for UDP-glucose pyrophosphorylase
Whsl5g15	500	2.3-2.9	*T. aestivum* 18S rRNA gene, partial se.
Whsl6l21		2.5-3.0	Unknown
Whsl9f19		2.1-3.0	Unknown
Whsl13g09		3.0	Unknown
Whsl15h14		3.2	Unknown

Se: sequence

RT-PCR

RT-PCR was performed for 4 selected clones (Whe11p20, Whe23jo6, Whsl1k21, and Whsl5g10). For the clone Whe11p20, a reduction in thickness of the amplified band was seen in wxBD and waxy CS (Figure 1). For the other three clones tested, the amplification was seen, however no differences could be observed in the thickness of the band. Band thickness is likely to correspond to the number of copies at the original RNA sample from each genotype.



1 2 3 100bp marker

Fig. 1. RT-PCR of tested plant materials with primers specific to clone, Whe11p20. 1. wxBD, 2. waxy CS, 3. CS. After reverse transcription,cDNA was amplified with the primers (F- ctaccacctgtcggttt, R- agtatgagccccgtggacta) for 30 cycles. *Arrow*: the amplified fragment was 154bp in size

Northern hybridization

Northern hybridization confirmed the results of microarray for two clones, Whe11p20 and Whsl5g10. But the differential expression seen with Whsl14j21 could not be observed. In both Whe11p20 and Whsl5g10, a thicker band was seen in CS (Figure 2), confirming the presence of a higher number of copies of the relevant mRNA. On the other hand the difference in band thickness of Whsl5g10 was unclear between wxBD and waxy CS. The clone Whsl14j21, which showed a higher expression in CS than in the other two wheat types at microarray, did not make clear differences in northern hybridization. Unlike Whe11p20 and Whsl5g10, the clone Whsl14j21 produced three main bands in all three wheat types after hybridization with the labeled clone, thus the small size differences in each band could not be compared.

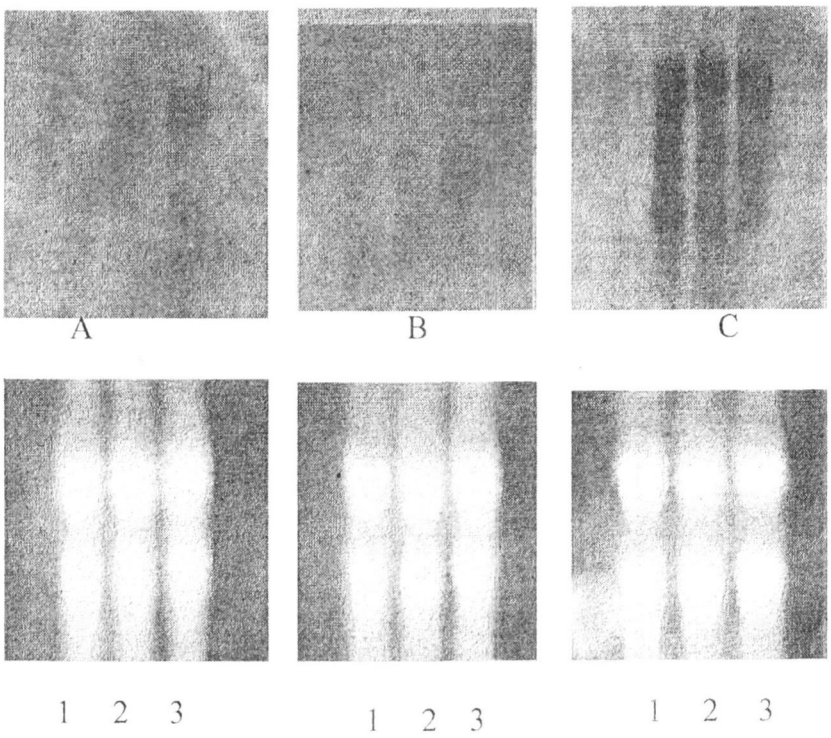


Fig. 2. **Northern hybridization of total RNA with clone (A) Whe11p20, (B) Whsl5g10 and (C) Whsl14j21. 5µg of total RNA of each genotype (lane 1. wxBD, 2. waxy CS, 3. CS) fractionated in 1% denaturing agarose gel (below), and hybridized with labeled probes at 60 °C (top).**

DISCUSSION

Since hexaploid wheat has three homoeologous *Wx* genes and, interaction among them is significant for amylose synthesis (Wickramasinghe and Miura, 2003), three near-isogenic genotypes were selected in this experiment as the initial step to screen ESTs related to amylose synthesis in wheat endosperm.

At 10 DPA, regardless the genotypes, the highest expression was shown in genes that code for storage protein subunits, for rRNA and for some functionally unknown clones. This agrees with Clarke *et al.* (2000) and Ogihara *et al.* (2003). Ogihara *et al.* (2003) analyzed the wheat ESTs on a large scale and listed the top 10 contigs most abundantly expressed in each cDNA library constructed from different tissues of wheat plant. The top 10 contigs in grains at 10 DPA were completely saturated by subunits of gliadin and glutenin. Sreenivasulu *et al.* (2002) analyzed genes specially expressed in embryo and pericarp of barley immature seeds at 1-7 DPA. RNAs expressed at high levels in the pericarp

mainly encode enzymes involved in carbohydrate and lipid metabolism, and in embryo it was mainly related to degradation and/or processing of proteins or starch. Weschke *et al.* (2003) evaluated the role of invertases and hexose transporters in maternal and filial tissues of barley caryopses. Therefore, it is interesting to identify those unknown clones functionally in order to study their effects on metabolic activities of developing seeds at the particular stage.

Out of the tested ESTs in the present experiment, only 1% showed more than 2-fold expression difference in Cy3 and Cy5 channels. This is comparatively a low level compared to the microarray data available in different plant species (Girke *et al.*, 2000; Endo *et al.*, 2002; Rossel *et al.*, 2002). The main reason would be the genetic background of the tested plant materials. All three genotypes used in this experiment are near-isogenic and have a CS genetic background in common (Miura *et al.*, 2002).

UDPase converts UDP-Glucose (UDP-Glc) into Glucose-1-Phosphate (Glc-1-P), which is directly transported or converted to ADP-Glucose (ADP-Glc) and then transported to plastids for starch synthesis. Therefore, UDP-Glc plays as a direct or indirect precursor of sucrose, starch and structural polysaccharides in plants (Sowokinos *et al.*, 1997). A higher level of expression (2.5 fold) of this enzyme was demonstrated in immature grains of CS than those of wxBD. However, the differences in the expression levels of this enzyme in wxBD and waxy CS were insignificant. The availability of ADP-Glc is one of the major factors limiting the amylose content (Clarke *et al.*, 1999). The ADP-Glc concentration that is required to saturate the GBSS I is much higher than that is needed for other starch synthases in developing pea embryos. It implies that if the ADP-Glc concentration were to fall, amylose synthesis would be reduced to a greater extent than would amylopectin synthesis. But, the effect of GBSS I (presence/absence or the concentration of it) on ADP-Glc or related enzymes of ADP synthesis are not understood.

The availability and effect of UDP-Glc on starch degradation has been intensively studied especially with respect to sweetening of potatoes during storage (Sowokinos *et al.*, 1997). It demonstrated that when the concentration of Glc-1-P increased to more than 0.2 mM, the affinity of the UGPase for the metabolic reaction decreased nearly 9-fold (Sowokinos *et al.*, 1993). The same phenomenon with opposite conditions would be one of the possible reasons for the increased level of this enzyme present in wheat endosperms with amylose. When both amylose and amylopectin are synthesized simultaneously in the endosperm, more and more precursor glucoses, Glc-1-P, are used and, this will trigger the production of more UDPase to make available Glc-1-P for starch synthesis.

Confirmation of microarray data by RT-PCR failed and there could be several reasons for this. One would be that the differential expressions shown by microarray data were insignificant to be reconfirmed by RT-PCR. The maximum difference at two Cy3 and Cy5 channels was about 7-fold (Whe11p20). The rest were in between 2 to 5-fold and, the tested clones by RT-PCR had 4.5-fold (Whe23j06), 3-fold (Whsl1k21) and 2.5-fold (Whsl5g10) differences at two fluorescent dye channels. It could also be due to the availability of gene families to tested clones. That could be possible as they were small in size (204bp to 619bp), and only known clone codes for ribosomal RNA. Furthermore all of them, except unknown clone, had homology to chloroplast DNA. In RT-PCR, non-specific

annealing was observed (data not shown). Some of them could not be eliminated even after optimizing PCR conditions, specially increasing annealing temperature.

Therefore, an attempt was made to prove microarray data through northern hybridization and the effort was successful. Whe11p20 is the clone, which shows the highest expression differences among tested wheat types. The expression was highest in CS followed by wxBD and waxy CS. Whsl5g10, which is a homolog of UDPase, showed expression difference only between CS and wxBD, but not between wxBD and waxy CS. Whsl14j21, an EST shows homology to the 26S proteasome regulatory particle triple-A ATP subunit 4 of rice, did not produce clear expression differences in the northern blot.

In this experiment, it was expected to reveal differential expression of starch synthesis genes in waxy and non-waxy wheat genotypes. Some of the genes related to starch synthesis [SSI (Whe10n02 and Whe4o16), SBE (Whe11m24 and Whe6a08) and a clone (Whe14o24) which is the transcript of *Wx-D1a* were found to be available in the generated arrays. However, none was observed either in the differential expression analysis or within most abundant transcripts. Further, no clones encoding DBE (de brancing enzymes) or ADPase, or genes of *Wx-A1a* or *Wx-B1a* were spotted in the arrays. One reason could be due to the technical limitation of microarrays. The array that was produced, although containing thousands of genes, may not contain a high representation of rarely expressed or "low-copy" genes. If the mRNA abundance is low, it is possible for those genes to be excluded from the cDNA library. Furthermore, the presence of many highly abundant transcripts, as those for seed storage proteins or other transcripts from house-keeping genes, has a dilution effect on low abundance transcripts. Thus, it is possible that cDNA microarray will continue to have difficulty in reliable detection of the most rarely expressed genes. The other reason would be that the clones of those genes were functionally not identified. There were 10 functionally unknown EST clones identified in this experiment. Therefore, it would be valuable to understand their functions on amylose synthesis.

CONCLUSIONS

The present study demonstrated the involvement of UDP glucose pyrophosphorylase and other ESTs on amylose biosynthesis in wheat endosperm. Since most of ESTs are unknown, further studies are needed to understand their role in amylose synthesis and to compare available information on starch biosynthesis in cereals.

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