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ORIGINAL PAPER

Identification of differentially expressed genes at two key endosperm development stages using two maize inbreds with large and small grain and integration with detected QTL for grain weight

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Abstract Maize endosperm accounts for more than 80% of the grain weight. Cell division and grain filling are the two key stages for endosperm development. Previous studies showed that gene expression during differential stages in endosperm development is greatly different. However, information on systematic identification and characterization of the differentially expressed genes between the two stages are limited. In this study, suppression subtractive hybridization (SSH) was used to generate four subtracted cDNA libraries for the two stages using two maize inbreds with large and small grain. Totally, 4,784 differentially expressed sequence tags (ESTs) were sequenced and 902 were non-redundant, which consisted of 344 unique ESTs. Among them 192 had high sequence similarity to the GenBank entries and represent diverse of functional categories, such as metabolism, cell growth/division, transcription, signal transduction, protein destination/storage, protein synthesis and others. The expression patterns of 75.7% SSH-derived cDNAs were confirmed by reverse Northern blot and semi-quantitative reverse transcription polymerase chain reaction, and exhibited the similar results (75.0%). Genes differentially expressed between two key stages for the two inbreds were involved in diverse physiological process pathway, which might be responsible for the formation of grain weight. 43.8% (70 of the 160 unique ESTs) of the identified ESTs were assigned to 39 chromosome bins distributed over all ten maize chromosomes. Eleven ESTs were found to co-localize with previous detected QTLs for grain weight, which might be considered as the candidate genes of grain weight for further study.

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Introduction

Endosperm is an important tissue from the point of view of breeders, because it is a major component of grain yield, and from the point of view of physiologists and evolutionists, because it provides the ground for seedling germination and initial growth (Méchin et al. 2007). Endosperm development has been well characterized at the cellular level. Cell division is characteristically involve in endosperm during 7–12 days after pollination (DAP), then endosperm cells enlarge and undergo several metabolic processes that result in the deposition of starch and storage proteins (Olsen 2004). During endosperm development, a complex gene expression system integrates carbohydrate, amino acid and storage protein metabolism (Arruda et al. 2000; Hunter et al. 2002). Some mutations specifically affecting the differentiation, development and metabolism of certain tissues or organs have been studied (Maizel and Weigel 2004; Tuteja et al. 2004), but the roles of most tissue-specific expressed genes remain unknown. Over 9 million expressed sequence tags (ESTs) from plant tissues are currently available at GenBank (http://www.ncbi.nlm.nih.gov/dbEST/dbEST_summary.html; December 5, 2008). The availability of large databases

of expressed genes offers a good opportunity to identify tissue-specific genes.

In maize (*Zea mays*), endosperm tissue is a major portion of the whole grain and the main storage site of starch and protein (Liu et al. 2008). Besides, it provides a useful model system to investigate the mechanism of endoreduplication and its physiological consequences in a seed storage tissue. Both studies based on analysis of ethyl methane sulfonate mutants (Neuffer and Sheridan 1980) and mutator transposon-induced mutants (Scanlon et al. 1994) suggested that at least 300 genes can cause a visible endosperm phenotype. But only few of these mutants have been molecularly characterized (Consonni et al. 2005). Otherwise, a higher number of genes are supposed to be expressed and play a role during endosperm development without being characterized by a visible mutant phenotype (Méchin et al. 2007).

Recently, cDNA libraries have been constructed at contrasted developmental stages, 4–6 and 7–23 days after pollination (DAP). Analysis of contigs and singletons suggested that at least 5,000 different genes could be expressed, excluding storage protein genes (Lai et al. 2004). This number was considered a minimal estimate following the work of Verza et al. (2005). Woo et al. (2001) analyzed cDNA libraries from developing endosperm of maize inbred line B73 to evaluate the expression of storage protein genes. Guo et al. (2003) have taken a genomic approach to examine global gene expression in the maize endosperm in relation to dosage and parental effects. Verza et al. (2005) generated 30,531 high quality sequence-reads from the 5′-ends of cDNA libraries from maize endosperm harvested at 10, 15 and 20 DAP. A further 196,900 maize sequence-reads retrieved from public databases have been added to this endosperm collection to generate MAIZEST, a database with tools for data storage and analysis. After sequence analysis using overlapping parameters, a subset of 2,403 assembled sequences was functionally annotated and revealed a wide variety of putative new genes involved in endosperm development and metabolism.

However, there have been no researches on the differentially expressed genes related to the cell division and deposition of reserves simultaneously. Thus, a more complete comparison of gene expression profiles should be meaningful, which might be able to formulate the molecular model of endosperm development. Suppression subtractive hybridization (SSH), termed PCR-based cDNA subtraction method, is such a useful technology that offers a high-throughput analysis of differential gene expression. Moreover, the modified procedure can overcome the problems of differences in mRNA abundance by incorporating a hybridization step that normalizes the sequence abundance during the course of subtraction by standard hybridization kinetics.

In the present study, SSH was used to generate a large collection of differentially expressed genes between two key endosperm development stages using two maize inbreds, one dent inbred Dan232 with large-size grain and one popcorn inbred N04 with small-size grain. Totally 4,784 differentially expressed ESTs were sequenced and 902 were non-redundant, which consisted of 344 unique ESTs. And the expression patterns of randomly selected ESTs were analyzed by semi-quantitative reverse transcription polymerase chain reaction (RT-PCR). The different expressed ESTs were located on the maize chromosomes by in silico mapping and eight ESTs from dent inbred Dan232, three ESTs from popcorn inbred N04 were found to co-localization with previous detected QTL for grain weight. To further study of these genes will provide new insight into the mechanisms of maize endosperm development and the formation of the grain weight.

Materials and methods

Kernel collection

A dent maize inbred Dan232 with large-size grain and a popcorn inbred N04 with small-size grain were planted at the Scientific Research and Education Center of Henan Agricultural University near Zhengzhou, Henan, China in 2006. Dan232 was derived from Lu 9 kuan × Dan340. N04 was derived from a Chinese popcorn variety BL03. Each plant was self-pollinated by hand. Ears were harvested at 3, 5, 7, 10, 15, 20, 25, 30 and 35 DAP, respectively. To increase the uniformity of the isolated kernels, the upper half and about one-sixth from the bottom of the ears were cut and discarded. From the remaining parts of the ears grains were isolated. Samples were collected from at least six ears and pooled at each time point. Some of the collected samples were frozen in liquid nitrogen immediately and stored at −70°C, others were used to measure grain fresh and dry weight.

Isolation of total RNA and mRNA

Total RNA was isolated from 10 to 20 DAP endosperms of the two inbreds using a hot phenol extraction (Kay et al. 1987). For PCR-select DNA subtraction, mRNA was purified from total RNA using an Oligotex™ mRNA Purification Kit (QIAGEN).

SSH and cDNA library construction

SSH was performed by using PCR-based cDNA subtraction kit (Clontech) according to the manufacturer's protocol. Four subtracted cDNA libraries, designated early stage

library (10 DAP) for N04, middle stage library (20 DAP) for N04, early stage library (10 DAP) for Dan232, and middle stage library (20 DAP) for Dan232, were constructed with enriched gene expressed specifically or at higher level in the 10 and 20 DAP endosperm of the two inbreds. Briefly, 2 μg of poly(A)$^+$ RNA from the tester and the driver (Table 1), were used for cDNA synthesis. The cDNAs were digested with *Rsa*I and ligated to different adaptors (Adaptor1: 5′-CTAATACGACTCACTATAG GGCTCGAGCGGCCGCCCGGGCAGGT-3′; Adaptor2R: 5′-CTAATACGACTCACTATAGGGCAGCGTGGTCGC GGCCGAGGT-3′). Two rounds of hybridization and PCR amplification were performed to enrich the differentially expressed sequences. The primary PCR was performed for 27 cycles with the following parameters: 94°C 30 s, 66°C 30 s, and 72°C 1.5 min. The secondary PCR was amplified for 12 cycles with the same parameters and the PCR products were directly ligated into the T/A cloning vector pGEM®-T Easy Vector (Promega) and transformed into Escherichia coli DH5α cells.

Amplification of cDNA inserts

A total of 2,500 transformants were randomly selected from each subtracted library to amplify the inserted sequences. The clones were grown overnight in 400 μl LB-Amp medium in a 96-well plate at 37°C. The cDNA inserts were amplified by PCR (PTC 200) using nested PCR primer 1 and 2R provided in the PCR-selected cDNA subtraction kit, which were complementary to sequences flanking both sides of the cDNA insert. Each reaction tube contained 2.5 μl 10× Ex *Taq* Buffer, 2 μl MgCl (25 mM), 2 μl dNTP (2.5 mM each), 1 μl of nested primer 1 and nested primer 2R (10 μM), 16.375 μl of PCR-grade water, and 0.125 μl TaKaRa Ex *Taq*. PCR was performed according to the following parameters: 95°C for 30 s and 25 cycles at 95°C for 10 s and 68°C for 2 min. The PCR products were electrophoresed on a 1.2% agarose gel to confirm the amplification quality and quantity.

Differential screening of subtracted cDNA library

The PCR products containing a cDNA fragment were denatured with 0.6 M NaOH and 1 μl of each fragment was dotted onto Four Hybond N$^+$ membranes (Amersham, UK). Each membrane consisted of a maximum of 96 dots. Serial dilutions of the whole population of cDNA fragments recovered from forward or reverse SSH steps were also included in the arrays as internal controls. The membranes were neutralized with Tris–HCl and baked under a vacuum at 80°C for 2 h and stored in plastic wrap until hybridization. The four membranes corresponded to four probes: two subtracted cDNA probes (forward and reverse subtracted) and two unsubtracted probes (10 and 20 DAP endosperm). The hybridization signal intensity was scanned using the software of Quantity one 4.30 (BioRad, Hercules, CA, USA) and then standardized by actin. Clones that had relatively strong signals when hybridized with a probe of positively subtracted cDNA were selected.

Sequence analysis

The selected positive clones were all single-pass sequenced by 3730 Automatic DNA Sequencer (ABI Prism, USA). Unique ESTs were selected using the Stackpack program. All unique ESTs were annotated on the basis of the existing annotation of non-redundant databases at the NCBI using BLASTN and BLASTX. Homologies that showed *E* value $<1 \times 10^{-10}$ with more than 100 nucleotides were considered significant. Functional classification of the ESTs was carried out according to the functional categories of Arabidopsis proteins (http://mips.gsf.de/proj/thal/db/index.html).

Semi-quantitative RT-PCR analysis

Two micrograms total RNA of each sample was used for first-strand cDNA synthesis in 20 μl reactions. 2.4 μl OligodT, 2.6 μl RNase Free ddH_2O, 70°C, 5 min; 4 μl M-MLV 5× Reaction Buffer, 0.5 μl RNase Inhibitor, 2 μl

Table 1 Four subtractive libraries of endosperm for the two inbreds at two key developmental stages

Subtractive library	Tester	Driver	Enriched genes in the library
Early stage library (10 DAP) for popcorn inbred N04	10 DAP endosperm cDNAs of N04	20 DAP endosperm cDNAs of N04	N04 endosperm specific expressed at 10 DAP
Middle stage library (20 DAP) for popcorn inbred N04	20 DAP endosperm cDNAs of N04	10 DAP endosperm cDNAs of N04	N04 endosperm specific expressed at 20 DAP
Early stage library (10 DAP) for dent inbred Dan232	10 DAP endosperm cDNAs of Dan232	20 DAP endosperm cDNAs of Dan232	Dan232 endosperm specific expressed at 10 DAP
Middle stage library (20 DAP) for dent inbred Dan232	20 DAP endosperm cDNAs of Dan232	10 DAP endosperm cDNAs of Dan232	Dan232 endosperm specific expressed at 20 DAP

dNTP Mixture (10 mM each), 1 μl M-MLV Reverse Transcriptase, 5.5 μl RNase Free ddH_2O, 42°C, 1 h; 70°C, 10 min. Gene-specific RT-PCR primers for the 12 differentially expressed cDNA fragments were designed according to the cDNA sequences by Primer 5.0 software (Supplementary table 1). Each reaction tube contained 2.5 μl 10× PCR Buffer, 2.5 μl dNTP Mixture (2.5 mM each), 1.0 μl Forward Primer (10 μm), 1.0 μl Reverse Primer (10 μm), 0.125 μl Takara La *Taq*, 1.0 μl cDNA, 16.875 μl ddH_2O. The thermal cycling parameters: 94°C for 1 min; 94°C for 40 s, 50°C-60°C for 40 s, 72°C for 1 min, 35 cycles; 72°C for 7 min. A 202 bp *β*-actin gene fragment was amplified as a positive control using the primer pair 5′-CGATTGAGCATGGCATTGTCA-3′ and 5′-CCCACTAGCGTACAACGAA-3′. The RT-PCR products were sequenced to verify the specialty of PCR amplification.

In silico mapping and co-localization

In silico mapping was based on anchored EST and BAC/BAC-end sequences (Xiao et al. 2007). All unique ESTs were compared to the mapped ESTs (http://www.maizegdb.org) via tblastx at *E* value $<10^{-20}$ to search for those highly homologous ESTs. A unique EST was considered locating at the same locus as its highly homologous cognate EST. Unmapped ESTs were compared with BAC (sequenced BAC and BAC-end) sequences via tblastx search at *E* value $<10^{-20}$. The highly homologous cognate BAC clones were used to find their corresponding fingerprint contigs (FPCs) in the website http://www.genome.arizona.edu/fpc/WebAGCoL/maize/WebFPC/. The SSR markers on FPCs flanking the BACs were identified and subsequently used to anchor the corresponding ESTs into the existing SSR linkage map.

The marks of QTLs for maize grain weight detected with different genetic maps were submitted to the Maize GDB (http://www.maizegdb.org) to get the physical position of the QTLs. Co-localization could be found by comparing the mapping information of ESTs to the physical position of the QTLs.

Results

Determination of the key development stages of endosperm

Following pollination, the fresh weight and dry weight of the whole kernel were quantified at 3, 5, 7, 10, 15, 20, 25, 30 and 35 DAP, endosperm, embryo and pericarp were quantified at 10, 15, 20, 25, 30 and 35 DAP for the two inbreds. As shown in Fig. 1, the dry and fresh weight of the whole grain increased slowly before 10 DAP, followed a fast increase in 10–20 DAP, and then increased slowly again for both inbreds. The whole grain weight of the two inbreds was different significantly. The tendency was the same for endosperm, embryo and pericarp (Fig. 2). The proportion of the endosperm in the whole grain dry weight increased rapidly between 10 and 20 DAP, and then increased slowly for both inbreds (Fig. 3). And endosperm accounted for more than 80% of the whole grain weight, while embryo and pericarp accounted for <20% after 30 DAP.

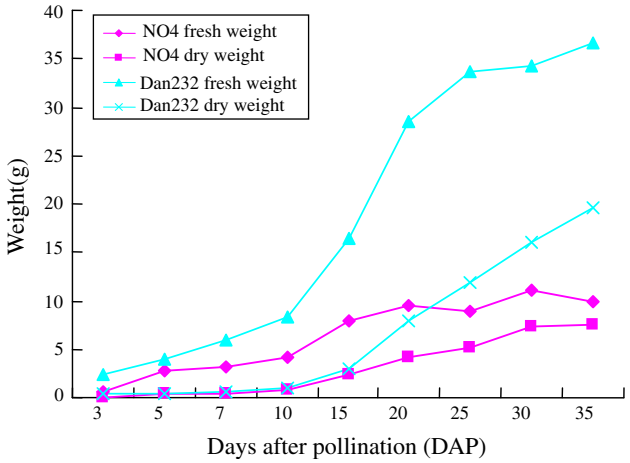

Fig. 1 Dynamic accumulation of fresh and dry grain weight for the two inbreds

Endosperm microstructures at 3, 5, 7, 10, 15, 20 and 25 DAP for the two inbred lines showed that 10 DAP endosperms were in the stage of cell division, then endosperm cells accumulated substances rapidly (Fig. 4). At 20 DAP, endosperm cells were filled with starch grains. The endosperm structure was also different between the two inbreds, hard endosperm for inbred N04 and soft endosperm for inbred Dan232, respectively. The starch granules in the endosperm of Dan232 was mostly spherical and loosely packed, while those in the endosperm of N04 was mostly polygonal and densely packed. Accordingly, 10 and 20 DAP were decided as two key stages for further study on the differentially expressed genes related with cell division and deposition of reserves during endosperm development.

Library construction and differential screening

In order to capture a wide spectrum of differentially expressed genes in maize endosperm development, four SSH libraries were constructed (Table 1). To further test the reliability of SSH and compare the expression pattern of differentially expressed ESTs between 10 and 20 DAP endosperm, reverse Northern blot analysis was conducted

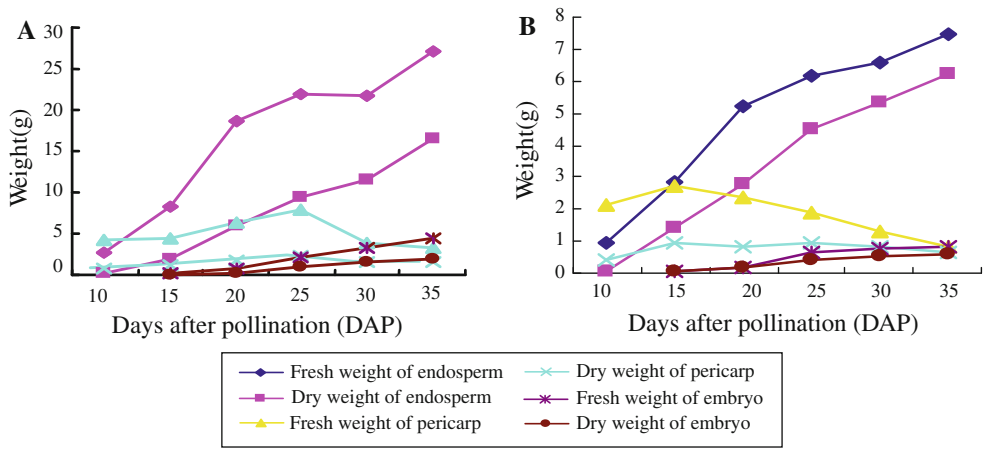

Fig. 2 Dynamic accumulation of fresh and dry weight of endosperm, pericarp and embryo for N04 (**a**) and Dan232 (**b**)

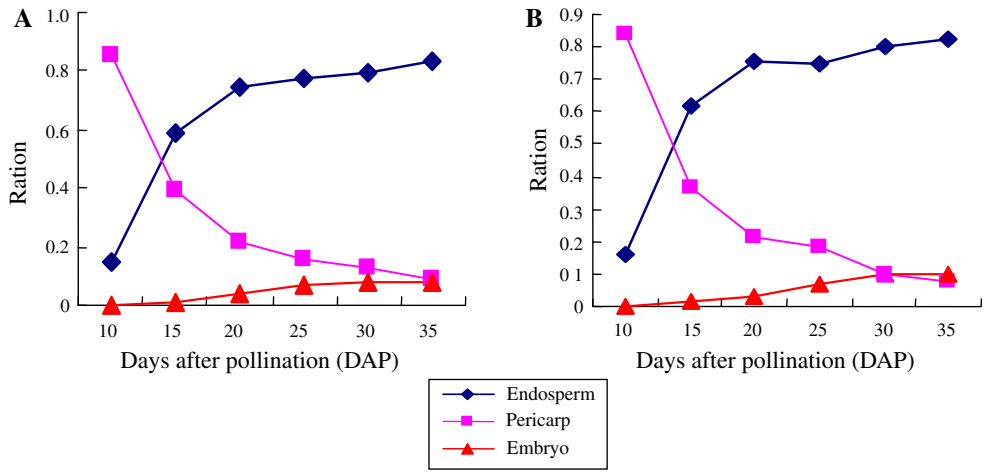

Fig. 3 Proportion of dry weight of endosperm, pericarp and embryo in the whole dry weight of grain for N04 (**a**) and Dan232 (**b**)

for a subset of 6,323 ESTs obtained after PCR screening of 10,000 clones from the four libraries. Reverse Northern blot analysis indicated that 765 out of the 1,049 ESTs tested from the early stage library for N04, 1,411 out of 1,862 ESTs from the middle stage library for N04, 1,014 out of 1,265 ESTs from the earlier stage library for Dan232 and 1,594 out of 2,147 ESTs from the middle stage library for Dan232 were differentially expressed, resulting a 72.9, 75.8, 80.2 and 75.7% efficiency for characterizing differentially expressed genes, respectively. Among the whole 4,784 differentially expressed ESTs, 3,005 (62.8%) and 1,779 (37.2%) ESTs showed up and down expression patterns, respectively.

Identification and classification of differentially expressed genes

After PCR screening and reverse Northern blot analysis, 4,784 differentially expressed ESTs from the four subtraction libraries were selected for further analysis. The cDNA insert size ranged from 100 to 1,000 bp, mostly between 200 and 500 bp. BLAST search indicated that 902 (18.9%) ESTs were non-redundant, with 147 ESTs from the early stage library for N04, 248 ESTs from the middle stage library for N04, 160 ESTs from the early stage library for Dan232 and 277 ESTs from the middle stage library for Dan232, respectively (Table 2). These non-redundant ESTs could be clustered into 344 ESTs, of which 140 were singletons and 204 were contigs made up of 2–20 overlapping clones.

Based on the BLAST search results, these 344 ESTs could be classified into three groups. The first group consisted of 192 ESTs with high similarity (BLASTX expectation values [*E*] of $<10^{-10}$) to database entries, suggesting that they are either the same gene or belong to the same gene family as those in the database. However, 47 (24.5%) ESTs show high similarity to unknown or hypothetical proteins. The second group, including 101 ESTs, had low sequence similarity (BLASTX expectation values [*E*] of $>10^{-10}$) to any database

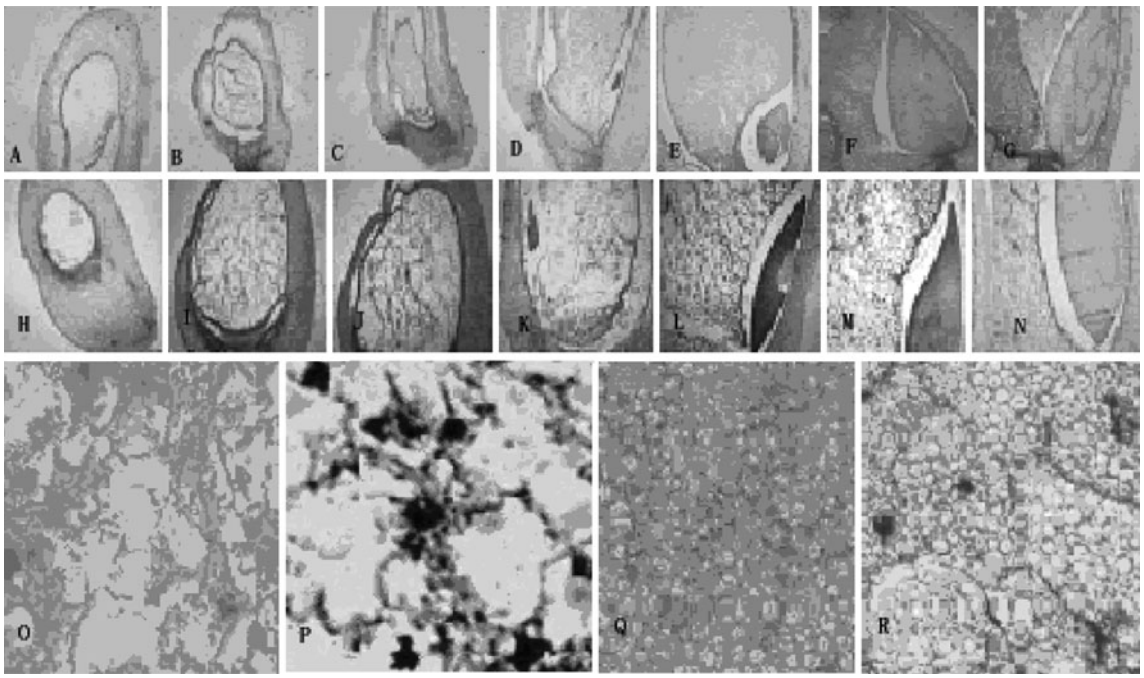

Fig. 4 The microstructure of endosperm during the grain developmental process

Table 2 The result of reverse Northern blotting and the basic information obtained in the four SSH libraries

SSH library	Northern blotting			Sequence analysis					
				No. of non-redundant	Unique ESTs		Amino acid comparability		No comparable result
	No. of difference	No. of no difference	No. of no signal		Contigs	Singletons	High	Low	
Early stage library for N04	765	197	87	147	46	24	31	26	13
Middle stage library for N04	1,411	284	167	248	54	35	55	23	11
Early stage library for Dan232	1,014	175	76	160	35	40	35	29	11
Middle stage library for Dan232	1,594	371	182	277	69	41	71	23	16
Total number	4,784	1,027	512	902	204	140	192	101	51

entries. The remaining 51 ESTs had no homologies with any genes in the database, which might represent either previously uncharacterized sequences or fragments that were too short to reveal any significant identity.

The 192 ESTs with high sequence similarity to the GenBank entries were selected for functional classification using Gene Ontology (GO) classification scheme (http://www.geneontology.org) and Panther classification scheme (http://www.pantherdb.org). These 192 ESTs represented a large range of functional categories, which included metabolism, cell growth and maintenance, signal transduction, response to stress, transcription regulation and others. Further classification and comparison of ESTs from the four different SSH libraries were listed in Table 3.

Except duplicate ESTs among the four SSH libraries, 160 unique ESTs were obtained. High proportion (71.9%) of these differentially expressed ESTs is involved in diverse metabolism pathways. The largest subset (21.9%) was related to protein destination/storage pathways, followed by carbohydrate metabolism (10.0%), protein metabolism (6.3%) and transporters (6.3%) (Fig. 5).

Comparison of differentially expressed genes at different developmental stages of endosperm

At the early developmental stage of endosperm, 145 unique ESTs were up-regulated expression, among which 75 were from Dan232 and 70 were from N04. High proportion (45.5%) of these ESTs is involved in diverse metabolism pathways, with 35 from Dan232 and 31 from N04. Nine ESTs were found encoding the same proteins, which were involved in metabolism, transcription, cell growth/division

Table 3 Functional classification of ESTs from the four SSH libraries

Putative function	Earlier stage library for N04	Middle stage library for N04	Earlier stage library for Dan232	Middle stage library for Dan232	Total
Metabolism	6	9	7	17	39
Transcription	3	1	3	3	10
Signal transduction	1	2	2	0	5
Cell growth/division	4	0	3	0	7
Protein destination/storage	2	23	3	22	50
Transporters	2	4	1	3	10
Protein synthesis	4	1	1	5	11
Cell structure	0	0	1	0	1
Disease/defense	1	3	1	3	8
Energy	2	0	1	0	3
Transposon	0	0	0	1	1
Unclear classification	2	3	4	5	14
Unknown protein	4	9	8	12	33
Total	31	55	35	71	192

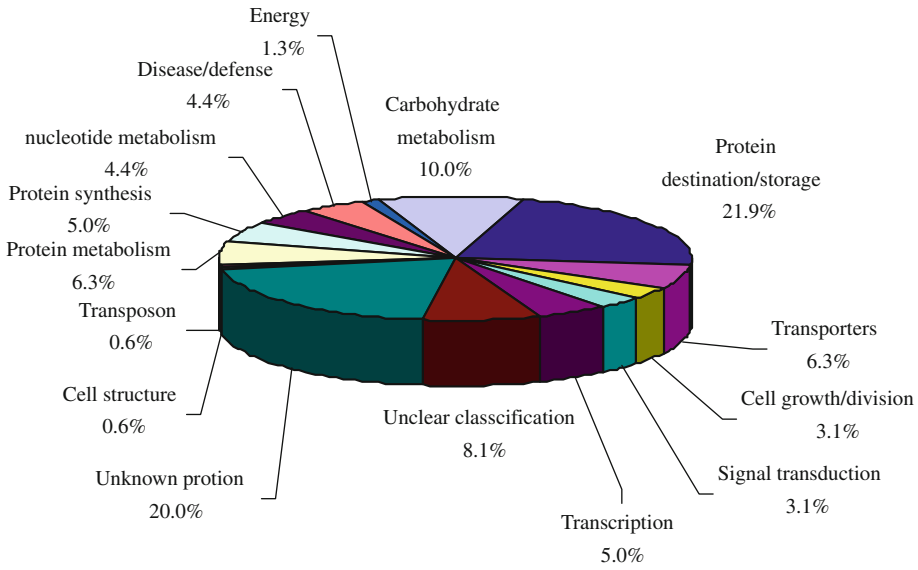

Fig. 5 Functional categories of putative differentially expressed ESTs

and energy functional categories (Fig. 6). Except the unknown or unclear functional category, the largest subset of ESTs from Dan232 (20.0%) and N04 (19.4%) were all related to metabolism, including protein, nucleotide and carbohydrate metabolism. These included genes for a homologue of succinate dehydrogenase, aconitase/aconitate hydratase, allene oxide cyclase, ATP synthase beta chain 2 and uncleaved legumin-1 in inbred Dan232, and genes for a homologue of SNF2-related domain containing protein, zinc ion binding protein, cytokinin oxidase 3 and malate dehydrogenase 5 in inbred N04. ESTs for transcription, cell growth/division and protein destination/storage were all 8.6%, and those for signal transduction were 5.7% in inbred Dan232. For inbred N04, the second largest functional category was cell growth/division and transporters, both of which was 12.9%, followed by transcription (9.7%), protein destination/storage (6.5%) and protein synthesis (6.5%). The different ESTs related to cell growth/division were glutathione S-transferase 4 in Dan232, mRNA for proliferating cell nuclear antigen (PCNA) and alpha 5 subunit of 20S proteasome in inbred N04. Both of the two inbreds had ESTs for a homologue of glycine-rich protein in maize and cell division control protein 48 homolog *E* in *Arabidopsis thaliana*.

At the middle development stage of endosperm, 199 unique ESTs were up-regulated expression, among which 110 and 89 ESTs were from Dan232 and N04, respectively. Also high proportion (51.76%) of differentially expressed ESTs were involved in diverse metabolism pathways, with 71 ESTs from Dan232 and 55 ESTs from N04. 23 ESTs were found encoding the same proteins, among which 14 ESTs were zein proteins, others involved in metabolism, protein synthesis and disease/defense functional categories.

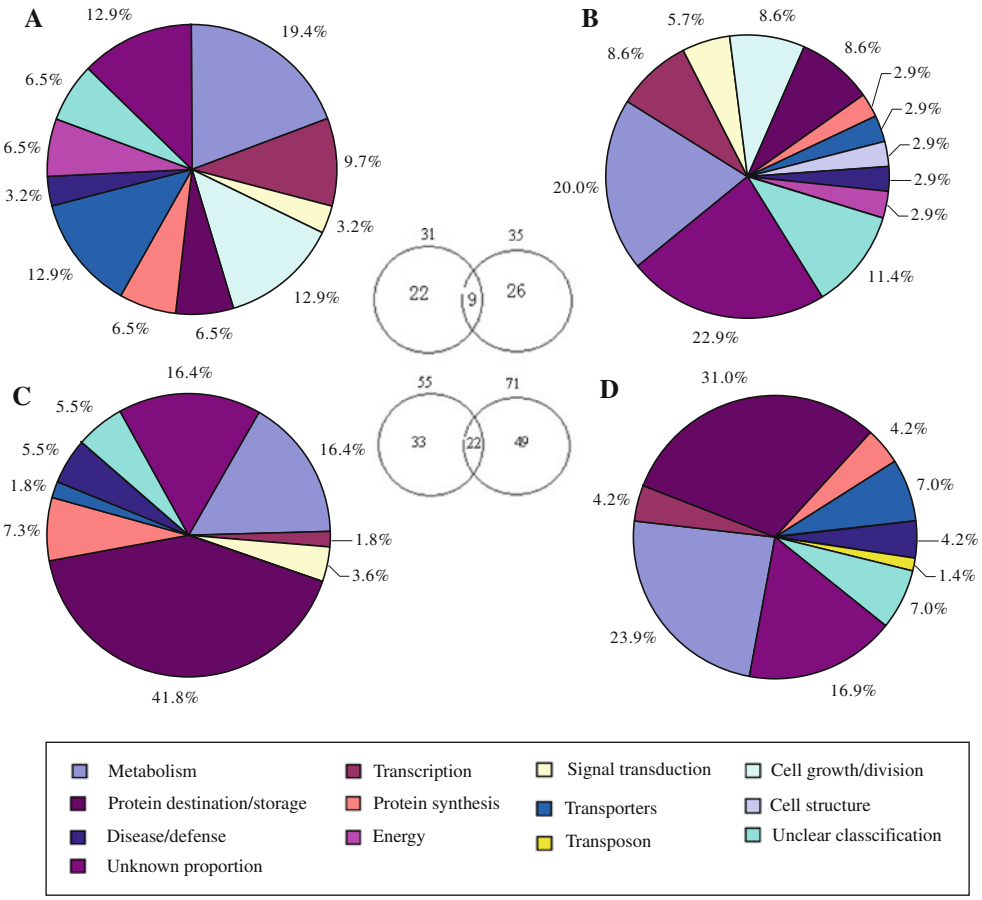

Fig. 6 Functional catalogues of putative differential expressed ESTs from the same developmental stage of the endosperm for the two inbreds and comparison between the two stages for the same inbred. **a** Early stage library for N04, **b** early stage library for Dan232, **c** middle stage library for N04, **d** middle stage library for Dan232

The largest subset of ESTs from inbred Dan232 (31.0%) and N04 (41.8%) were related to protein destination/storage, and they were all storage protein. The second largest functional category from inbred Dan232 (23.9%) and N04 (16.4%) were all related to metabolism. These included genes for a homologue of pyruvate, orthophosphate dikinase 1, peroxidase 57, aminotransferase AGD2, pyruvate decarboxylase, alanine aminotransferase, acetoacetyl CoA thiolase and several starch synthesis-related genes in inbred Dan232, and ESTs for a homologue of ribosomal protein, 3-phosphoinositide dependent protein kinase 1 (PDK1), heat shock protein 91 (HSP91), tryptophan synthase beta-subunit (TSB1) and several starch synthesis-related genes in inbred NO_4. One EST encoding cation-chloride co-transporter (CCC) protein was up-regulated both in the early stage for N04 and in the middle stage for Dan232.

Confirmation of SSH expression patterns by semi-quantitative RT-PCR analysis

PCR-selected cDNA subtraction is a powerful tool for identifying differentially expressed genes. However, subtractive PCR products might contain ESTs that are not truly differentially expressed between hybrid and its parents. Semi-quantitative RT-PCR was further applied to validate the expression pattern of 12 selected ESTs with putative functions involved in a diverse set of biological pathways. Among them, four ESTs (PE12C5, PE15C3, PE21F3 and PE24D9) derived from the early stage library for inbred N04, two (PM11F7 and PM17E5) from the middle stage library for inbred N04, two (DE7A10 and DE24D3) from the early stage library for inbred Dan232 and four (DM17F9, DM2A4, DM29A6 and DM24B11) from the middle stage library for inbred Dan232. According to RT-PCR results, eight ESTs (PE12C5, PE15C3, PE24D9, DE7A10, DE24D3, PM11F7, DM2A4 and DM29A6) showed the same differential expression patterns as SSH and reverse Northern results, and two (DM17F9 and DM24B) showed different differential expression pattern. Another two ESTs (PE21F3 and PM17E5) failed to show expression difference between the early and the middle stages in endosperm development on RT-PCR.

Mapping and annotation of differentially expressed genes

In order to identify genes in association with grain weight in maize, the ESTs isolated by SSH were located on the

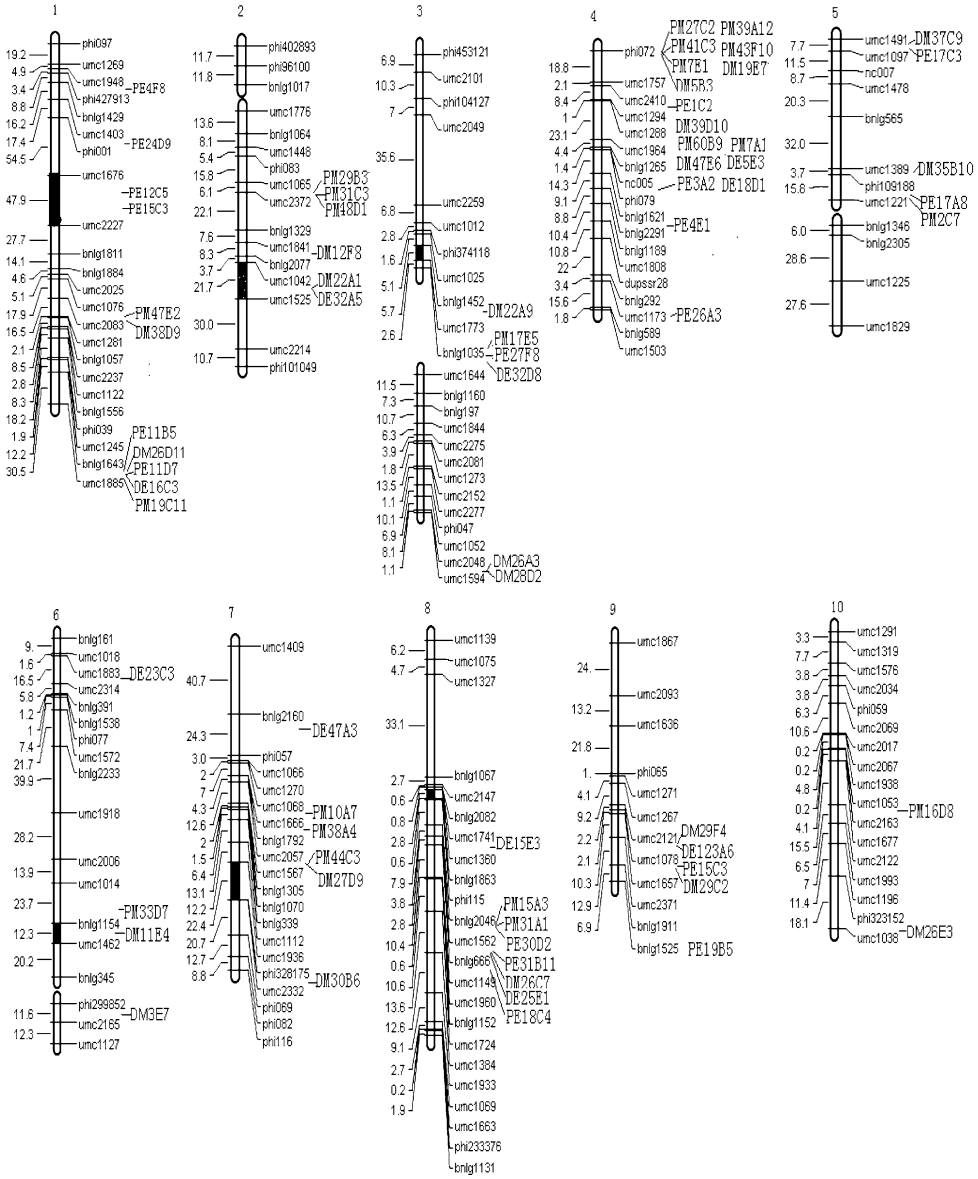

Fig. 7 Integration of ESTs and detected QTL for grain weight in the genetic map constructed using $F_{2:3}$ families developed from Dan232 × N04. *Black bars* represent the region of detected QTL for grain weight

maize chromosomes by in silico mapping and co-localization with grain weight QTL which has been detected in previous researches. Altogether 70 of the 160 unique ESTs were assigned to 39 chromosome bins distributed over all 10 maize chromosomes, accounting for 43.8%. And these 70 ESTs were marked on the genetic map constructed using $F_{2:3}$ families developed from the same two inbreds as in this study in our laboratory (Li 2005; Li et al. 2007) (Fig. 7). 17 of the 70 ESTs (24.3%) were located on chromosome 4, with 11 ESTs (15.7%) on chromosome 1, 8 ESTs on chromosome 8, all 6 ESTs on chromosome 2, 3 and 7, and 2 ESTs on chromosome 10.

QTL mapping for grain weight has been thoroughly done in previous researches (Stuber et al. 1987; Veldboom and Lee 1994; Doebley et al. 1994; Schön et al. 1994; Goldman et al. 1994; Berke and Rocheford 1995; Austin and Lee 1996, 1998; Melchinger et al. 1998; Austin et al. 2000; Xiang et al. 2001; Yang et al. 2005; Lan et al. 2005; Xiao et al. 2005; Li 2005; Li et al. 2007; Song 2003; Willmot et al. 2006; Yan et al. 2006; Wang et al. 2007a; Tang et al. 2007; Wassom et al. 2008). Comparing with 271 QTL previously detected for grain weight, 11 mapped ESTs (15.7%) were located in the same marker intervals as the detected QTL (Table 4), which were located on chromosome 1, 2, 3, 6, 7 and 8, respectively. Nine ESTs (PE12C5, PE15C3, DM22A1, DE32A5, DM22A9, DM26A3, PM44C3, DM27D9 and DM11E4) were encoding zinc finger (C3HC4-type RING finger) family, GTP binding protein, glucose and ribitol dehydrogenase, alpha-tubulin, preprotein translocase secA subunit, ADP-glucose pyrophosphorylase large subunit and pyruvate, opaque2 (O_2) modifier, 16 kDa gamma zein and orthophosphate dikinase. Two ESTs (DM30B6 and

Table 4 The result of integration for the locations of ESTs and QTL detected for grain weight

EST	Chrom.	Marker interval	Bin	Previous studies on QTL detection	Library source	Predicted function for the ESTs
PE12C5	1	umc1676–umc2227	1.04–1.05	Li (2005)	Early stage library for N04	Zinc finger (C3HC4-type RING finger) family protein
PE15C3	1	umc1676–umc2227	1.04–1.05	Li (2005)	Early stage library for N04	GTP binding protein
DM22A1	2	umc10–bnl8.44b	2.08	Schön et al. (1994)	Middle stage library for Dan232	Glucose and ribitol dehydrogenase
DE32A5	2	umc10–bnl8.44b	2.08	Schön et al. (1994)	Early stage library for Dan232	Alpha-tubulin mRNA
DM22A9	3	umc175	3.04	Austin and Lee (1996)	Middle stage library for Dan232	Preprotein translocase secA subunit
DM26A3	3	SH2	3.09	Berke and Rocheford (1995)	Middle stage library for Dan232	ADP-glucose pyrophosphorylase large subunit gene
DM11E4	6	umc21–bnl3.03	6.05	Schön et al. (1994)	Middle stage library for Dan232	Pyruvate, orthophosphate dikinase
PM44C3	7	umc2057–umc1567	7.02–7.03	Li et al. (2007)	Middle stage library for N04	opaque2 Modifier
DM27D9	7	umc2057–umc1567	7.02–7.03	Li et al. (2007)	Middle stage library for Dan232	16 kDa gamma zein
DM30B6	7	bnl14.07–umc151	7.04–7.05	Schön et al. (1994)	Middle stage library for Dan232	*Zea mays* PCO128296 mRNA
DE15E3	8	bnl9.11–bnl9.44	8.02–8.03	Schön et al. (1994)	Early stage library for Dan232	*Oryza sativa* (indica cultivar-group) cDNA clone:OSIGCPI225A24

DE15E3) had high similarity with *Z. mays* PCO128296 and *Oryza sativa* (indica cultivar-group) cDNA clone OSIGCPI225A24. Among them, PE12C5 and PE15C3 were located at the same marker interval umc1676–umc2227 on chromosome 1, PM44C3 and DM27D9 were located at the same marker interval umc2057–umc1567 on chromosome 2 as the QTL for grain weight detected using the $F_{2:3}$ families developed from the same inbreds as in this study (Li 2005; Li et al. 2007). Two ESTs (DM22A1 and DE32A5) were located at marker interval umc10–bnl8.44b on chromosome 2, on which QTL for grain weight had been detected in one testcross population by Schön et al. (1994). DM11E4, DM30B6 and DE15E3 were located at marker intervals umc21–bnl3.03, bnl14.07–umc151 and bnl9.11–bnl9.44 on chromosome 6, 7 and 8, respectively. QTL for grain weight were also detected in one testcross population by Schön et al. (1994). Two ESTs (DM22A9 and DM26A3) on chromosome 3 were linked with the same marker umc175 and SH_2 as the QTL for grain weight by Austin and Lee (1996) and Berke and Rocheford (1995).

Discussion

Cell division and kernel filling represent the main biological processes in kernel development (Kiesselbach 1998). So they were the most important stages in the endosperm development. In this study, two developmental stages, 10 and 20 DAP, were chosen based on the dynamic fresh and dry weight of endosperm and the microstructure during grain development, which was consistent with previous reports (Kowles and Phillips 1985). Differentially expressed ESTs with cell growth/division functions were found in the 10 DAP libraries, while no ESTs with such functions were found in the 20 DAP libraries. Zein proteins were only isolated in the 20 DAP libraries. Clearly, this was consistent with the physiology of endosperm development. Therefore, the differentially expressed genes cloned at the two developmental stages in this study could reflect the real endosperm development, and could provide useful information to discover the molecular mechanism of endosperm development.

Cell division and expression of related genes

Cell division plays a crucial role in almost all aspects of growth and developmental processes in plants (Meijer and Murray 2001; Dewitte and Murray 2003). In yeast and animals, cell cycle regulation has been well characterized. In plants, it is less well characterized. However, many important genes have been identified during the past decade, and their functions and regulation mechanisms have begun to be understood (Vandepole et al. 2002; Dewitte and Murray 2003).

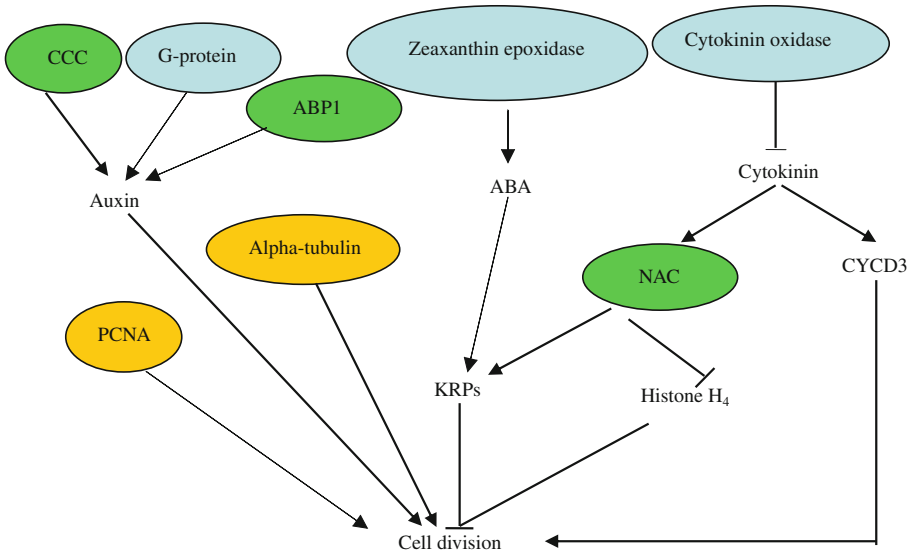

Fig. 8 Pathways of differentially expressed genes involved in cell division in endosperm development of maize. *Arrow* represents promotion of the process. *Bar* represents inhibition of the process

In this study, 10 DAP endosperms for both inbreds were in cell division stage, which could be supported by the fact that up-regulated genes related to the regulation of cell division were only cloned in the two early SSH libraries. Through sequence similarity search and gene expression study, a simple hypothesis on the regulation network for cell division in maize endosperm development could be proposed as in Fig. 8. It is well known that plant hormones are considered as key regulators to seed development (Davies 1987; Brenner and Cheikh 1995). The relationships among these genes could be considered with regard to three pathways: abscisic acid (ABA), cytokinin and auxin.

ABA signaling pathway

ABA plays a central role at least in higher plants by regulating plant growth and development (Zeevaart and Creelma 1988; Bray 1997). Exogenously applied ABA has been found to inhibit cell division in some plant tissue systems (Barlow and Pilet 1984; Saini and Aspinall 1982). In our results, one EST encoding zeaxanthin epoxidase was up-regulated expressed in 10 DAP in the endosperm of inbred N04. Zeaxanthin epoxidase has been shown to convert zeaxanthin to all-transviolaxanthin by a two-step epoxidation in the ABA biosynthetic pathway (Marin et al. 1996). One of the possible targets of ABA in the inhibition of the cell cycle is a cyclin-dependent protein kinase inhibitor (KRP1). This protein interacts with an A-type cyclin-dependent kinase (CDKA) and inhibits histone H1 kinase. In the embryo of apple, ABA inhibited the transition of nuclei to the G2 phase of the cell cycle, and, consequently, cell division was inhibited (Bouvier-Durand et al. 1989). Herein, it was proposed that the higher level of zeaxanthin epoxidase in the cell division stage for the small-size grain inbred N04 might lead to high levels of ABA, which could induce the expression of KRP1 gene (Wang et al. 1998), decrease the rate of cell division, and evenly limit the storage capacity in the grain. However, it has been reported that ABA content in large-size rice grains was higher than that in small-size rice grains during grain filling (Kato et al. 1993), and ABA content was positively correlated with grain filling rate at the early grain filling stage in wheat and rice (Bai et al. 1989; Wang et al. 1998; Yang et al. 1999). The function of ABA in maize grain filling should be an interesting question in further study.

Cytokinin signaling pathway

Cytokinins are generally found in the endosperm of developing seeds, which may be required for the cell division during the early phase of seed setting (Yang et al. 2000). Cytokinin oxidase irreversibly degrades cytokinins by cleaving the N6-side chain from the adenine/adenosin-emoiety. Larkins et al. (2001) considered that cytokinin oxidases seemed to play an important role in restricting cell division and regulate the sink capacity of the kernel. The molecular event verified concerning the effects of cytokinins on cell division is the induction of CYCD3-1, which is subsequently incorporated into the typical retinoblastoma pathway during cell cycling and accelerates cell division (Riou-Khamlichi et al. 1999). The activated NTM1 (NAC with transmembrane motif1) transcription factor enters the nucleus and induces a subset of CDK inhibitor genes (KRPs) and represses the histone H4 gene, resulting in reduced cell division. The promotion of cell division by CYCD3-mediated cytokinin signaling would be countered by the NAC-mediated induction of CDK inhibitors (KRPs) to maintain the cell division rate to an optimal level under a given growth condition (Kim et al. 2006).

In our present study, one EST encoding cytokinin oxidase was up-regulated expressed in 10 DAP endosperm of the small-size grain inbred N04, which might restrict the cell division in some degree. One EST encoding NAC transcription factor was up-regulated expressed in 10 DAP endosperm for both inbreds. For the large-size grain inbred Dan232, an EST encoding alpha-tubulin was also be cloned, which might play a crucial role in both cell elongation and cell division (Goddard et al. 1994). The effects of these genes in cell division of maize endosperm needs to be approved in further study.

Auxin signaling pathway

Auxins control cell division and cell elongation through different receptors by different pathways in plant development. Recently, response of cell division to auxin has been shown to require the activity of a putative heterotrimeric G-protein, whereas it was not dependent on this G-protein for cell elongation (Ullah et al. 2001). One auxin binding protein1 (ABP1) from maize being a receptor mediating auxin-induced membrane hyperpolarization could lead to a loosening of non-covalent bonds in the cell wall, allowing cell expansion (Barbier-Brygoo 1995; Rayle and Cleland 1992).

Herein, three ESTs were found up-regulated at early stage for the small-size grain inbred N04, which encoded a G-protein, a CCC and a PCNA, respectively. The ORF of this G-protein has been cloned (Liu et al. 2009), and further researches are being in progress. PCNA is critical for cellular DNA replication and cell division induced by many proliferating signals (Tsurimoto 1998; Fairman 1990; Celis et al. 1987). Previous research has shown that the C-termini of CCCs are functional subunits to promote auxin-independent triggering cell division (Hinrich et al. 1997). Since one EST encoding a CCC was also up-regulated at middle stage for the large-size grain inbred Dan232, it could be supposed that the transcription time of the CCC gene was different between the two maize inbreds, which might facilitate the regulation of reserve deposition in the large-size grain inbred Dan232. One EST encoding an ABP1 was up-regulated at middle stage for both inbreds, which might be related to the auxin-independent triggering cell division and cell expansion.

Deposition of reserves and expression of related genes

During endosperm development, large quantities of carbohydrates and proteins accumulate. Starch represents the majority of reserve products in maize endosperm, and comprises more than 65% of the seed dry weight in maize. In plant, the main function of sucrose synthase (SS) is to cleave sucrose to produce uridine diphosphate glucose (UDPG), which ultimately provides increased quantities of glucosyl for starch production. In our present study, one EST encoding starch synthase isoform zSTSII-2 (DM35A6) was up-regulated expressed at 20 DAP endosperm of large-size grain inbred Dan232, which might reflect its essential function in supplying raw materials for starch synthesis. In addition, other differentially expressed genes putatively encoding most enzymes for starch synthesis were also be found, including pullulanase-type starch debranching enzyme 1, UDP-glucose pyrophosphorylase (UGPase), ADP-glucose pyrophosphorylase (AGPase) large and small subunits. The expression of these genes and overrepresentation of starch and sucrose metabolism might imply that large quantities of starch accumulation occurred at 20 DAP. AGPase controls a rate-limiting step in the glycogen and starch biosynthetic pathway. Increased cytoplasmic AGPase activity has a significant effect on sink activity and, in turn, on seed weight in transgenic maize plants (Wang et al. 2007b). The ESTs encoded endosperm-specific shrunken2 and brittle2 were detected in the middle stage SSH library for the large-size grain inbred D232. Only one EST encoding UGPase was found in the middle stage SSH library for the small-size grain inbred N04. Accordingly, it could be proposed that the up-regulated expression of starch synthesis-related genes in the large-size grain inbred Dan232 promoted the starch biosynthetic pathway, and thus more starch might accumulate in the large-size grain inbred Dan232 than in the small-size grain inbred N04.

Endosperm is also the major site for storage protein accumulation in the maize grain. Herein, a large quantity of zeins-related genes were found differentially expressed and greatly increased at 20 DAP endosperm for both inbreds, which was similar to the results of Thompson and Larkins (1989). Among the 14 zeins-related ESTs isolated in the two SSH libraries at middle stage for both inbreds, the 22- and 19-kDa zeins were the most. This was consistent with the fact that the 22- and 19-kDa zeins constitute 75–86% of the total zein fraction (Esen 1987). Five ESTs, ZSF4C1, 27 kDa γ-zein, 19 kDa alpha zein D2, O_2 modifier, 19 kDa alpha zein B2, were only detected up-regulated expressed at the middle stage for the small-size grain inbred N04. O_2 modifier genes convert the soft endosperm of an O_2 mutant to a hard and vitreous phenotype (Etti et al. 1993). The primary biochemical change associated with the expression of these genes is a two- to threefold increase in synthesis of the 27-kDa γ-zein storage protein. The mechanism by which γ-zein converts an opaque seed to a vitreous phenotype may involve cross-linkage of the protein through disulfide bridges (Lopes and Larkins 1991). In our present study, one EST encoding the thiol-disulfide exchange intermediate was found up-regulated at the

20 DAP endosperm in the large-size grain inbred Dan232, while it was not up-regulated in the small-size grain inbred N04.

Transcription factors and expression of related genes

The regulation of gene expression in eukaryotes mainly occurred at the transcription level. Transcription factors can mediate RNA polymerase II to transcript correctly from the promoter site, thereafter activate or inhibit the expression of related genes. Herein, ESTs encoding bZIP type transcription factor and ring zinc finger protein were found up-regulated expressed at 20 DAP endosperm in the large-size grain inbred Dan232. Onodera et al. (2001) reported that bZIP type transcription factor was highly expressed in aleurone and endosperm tissues and may be important in regulating gene expression in developing rice grains. O_2 was a bZIP type transcription factor gene, which regulated the expression of zeins as mentioned above. An EST encoding NAC transcription factor and an EST encoding MADS domain transcription factor were up-regulated expression at 10 DAP endosperm for both inbreds. An EST encoding another MADS domain transcription factor was up-regulated expressed at 20 DAP endosperm for the large-size grain inbred Dan232. And an EST encoding C3HC4-type ring finger was also found up-regulated expressed in the small-size grain inbred N04. Therefore, the MADS domain transcription factor up-regulated expression at 10 DAP for both inbreds might regulate the genes expression related with cell division. The MADS domain transcription factor up-regulated expressed at 20 DAP for the large-size grain inbred Dan232 might be importance during the stage of kernel filling.

Co-localization of differentially expressed genes with kernel weight QTLs

Positional cloning is one of the major approaches used to identify QTLs, but it is very laborious and time consuming (Shi et al. 2005). Up to now, there have been only a few reports in positional cloning of QTL in maize (Salvi et al. 2002; Wang et al. 2005; Zheng et al. 2008). In silico mapping may provide an alternative for pinpointing QTLs and facilitate the identification of candidate genes in maize (Shi et al. 2005).

In this study, among the 70 mapped ESTs, eight ESTs from the large-size grain inbred Dan232 and three ESTs from the inbred N04 were found to co-localize with grain weight QTLs. Clearly, much more differentially expressed ESTs for the large-size grain inbred Dan232 were related with QTL for grain weight. This is consistent with the hypothesis that differentially expressed genes derived from the genome of the large-size grain inbred Dan232 might be candidate genes for the previously mapped QTL for grain weight or at least be involved in grain development. And the most interesting thing was that one EST encoding AGPase large subunit, which was a rate-limiting enzyme in the glycogen and starch biosynthetic pathway, has been proved to increase seed weight (Wang et al. 2007b). Therefore, the AGPase large subunit could be considered as an important candidate gene for grain weight. Of course, such associations between candidate genes and grain weight need to be proved in further research. Detailed characterization of these genes will improve our understanding on the endosperm development in maize.

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