Identification of 17 differentially expressed cDNAs between wheat reciprocal cross-fertilized kernels and their parents

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Abstract

In order to provide an insight into molecular basis of cross-fertilized kernel advantage and heterosis, we performed screening of differentially expressed genes between reciprocal cross-fertilized kernels and their parents at 2, 6, and 12 days after pollination (DAP) by using differential display technique. Seventeen differentially expressed cDNAs were verified by reverse-northern blot. Sequence analysis and database search revealed that differentially expressed genes between reciprocal cross-fertilized kernels and their parents included genes involved in metabolism, signal transduction, transcription factor and so on. In silico expression analysis of the 9 differential expressed genes in crown, flower, leave, root, stem, inflorescence and seed tissues, and indicated that they are expressed in various tissue-specific patterns. These results indicated that diverse pathways may involve in wheat heterosis formation.

Keywords: Differential display; Gene expression; Heterosis; Seed development; Wheat

1. Introduction

Hybrid cultivars have been used commercially in many crop plants and have made significant contribution to the world food supply (Duvick, 1997). However, the molecular basis of heterosis is still an area that needs to be elucidated (Cheng et al., 1996; Sun et al., 1999). Although the genome in hybrid F\textsubscript{1} is derived from its parental inbreds, hybrid performance is quite different from its parental inbreds, the differential gene expression between hybrids and their parents should be responsible for the observed heterosis (Sun et al., 1999). In fact, high rates of RNA and protein synthesis in hybrid maize were reported in early experiments (Mino and Inoue, 1980; Nebiolo et al., 1983). Romagnoli et al. (1990) reported that that some proteins and mRNAs are differentially synthesized and expressed in root tips between hybrid F\textsubscript{1} and its parental inbreds. Tsafaris (1995) and Tsafaris and Kafka (1998) demonstrated that the mean mRNA quantity for 35 tested genes were higher in a highly heterotic hybrid than in a non-heterotic hybrid and their parents. Differential display technique (Liang, 1992) has been successfully used in detecting genes differentially expressed between hybrids and their parents in crops (Xiong et al., 1999; Sun et al., 1999).

Wheat hybrids have been shown to demonstrate heterosis in terms of biomass, productivity and better stability of performance over environments (Sun et al., 1996). Previous attempts to understand the mechanism of wheat heterosis were mainly focused on physiological and biochemical aspects, which demonstrated that wheat hybrids were different from their parental inbreds in many biochemical properties, such as mitochondrial oxidation and phosphorylation, nucleic acid synthesis, phytohormone levels and enzyme activities (Liang et al., 1993; Tang et al., 1993). Recently, by using differential display of mRNA, we analyzed the differential gene expression between two hybrids and their parents in both primary roots and seedling leaves, and detected differences in gene expression patterns between hybrids and their parents, and found that the differential expression patterns are also dependent on the tissues tested and developmental stages (Ni et al., 2000a,b; Sun et al., 2004), and some differential gene
expression patterns were correlated with heterosis (Xiong et al., 1999; Wu et al., 2001), cloning and characterization of differentially expressed genes between hybrids and their parents will provide further insight into understanding the molecular mechanism of heterosis (Ni et al., 2000a,b; Wu et al., 2003).

Seed development represents a unique transition state in the life cycle of higher plants, providing the physical link between parental and progeny sporophytic generation. Several studies have shown that the cross-fertilization advantage was observed during the early stage of embryo development and grain filling (Groszmann and Sprague, 1948; Cherry et al., 1961; Yamada et al., 1992; Bulant and Gallais, 2000). However, gene expression between reciprocal cross-fertilized kernels and their parents remains to be revealed. Therefore, it was reasonable to speculate that change in genes expression should be the primary determinant for heterosis.

In this study, we conducted a differential display analysis of reciprocal cross-fertilized kernels and their parents during the early stage of development. Seventeen differentially expressed cDNA fragments between reciprocal cross-fertilized kernels and their parents at 6 DAP were cloned and sequenced, their expression pattern were investigated and possible roles in heterosis were discussed.

2. Materials and methods

2.1. Preparation of plant materials

Field grown plants of wheat line Nongda3159, Jingdong 6, Nongda8790 and Nongda3214 were used as female respectively, and emasculated before anthesis to avoid uncontrolled fertilization. At anthesis, some plants were self-fertilized and others were hybridized and produced reciprocal cross-fertilized combinations Nongda3159 × Jingdong6, Nongda3159 × Yuandong8790, Nongda3159 × Nongda3214, respectively. Previous field tests had demonstrated that the three hybrids show different levels of heterosis, highly heterotic combination Nongda3159 × Jingdong6, heterotic combination Nongda3159 × Yuandong8790, non-heterotic combination Nongda3159 × Nongda3214, yielded 15%, 10% and 5% more than the higher-yielding parent in terms of grain yield per plant, respectively (our unpublished data). The self-fertilized and reciprocal cross-fertilized kernels were collected at 2, 6, and 12 day after pollination and stored at −80 °C.

2.2. RNA extraction

The kernels were ground to fine powder in liquid nitrogen and transferred into 5 ml Eppendorf tubes. Total RNA was isolated from prepared samples using the RNasea kit (Sangon, Shanghai, China) according to the manufacturer’s instruction and then subjected to DNase digestion in the presence of ribonuclease inhibitor. Then, the RNA samples were extracted with phenol: chloroform (1:1) and chloroform respectively. Finally, precipitated with ethanol and the pellet was dissolve in ddH₂O treated by DEPC.

2.3. Reverse transcription

Equal amounts of 2 μg of total RNA was reverse-transcribed into cDNA in a 20 μl reaction system, containing 50 mM Tris–HCl (PH8.3), 75 mM MgCl₂, 10 mM DTT, 50 μM dNTP, 200 U MMV reverse transcriptase (Promega) and 50 pmol either one base anchor oligonucleotides HT₁₁A, HT₁₁C, or HT₁₁G. Reverse transcription was performed for 60 min at 37 °C with a final denaturation step at 95 °C for 5 min.

2.4. PCR amplification of cDNA

The following primers were synthesized according to Von der Kammer et al. (1999).

3’ end anchored primers
HT₁₁A: 5’-AAGCAGCTTTTTTTTTTA-3’; HT₁₁C: 5’-AAGCAGCTTTTTTTTTTC-3’;
HT₁₁G: 5’-AAGCAGCTTTTTTTTTTG-3’.
5’ end oligonucleotide primers
DD18: 5’-TGCCGAAGCTTTGTCAC-3’; DD19: 5’-TGCCGAAGCTTTGTCAG-3’;
DD20: 5’-TGCCGAAGCTTTGTCAT-3’; DD23: 5’-TGCCGAAGCTTGATTCCG-3’;
DD32: 5’-TGCCGAAGCTTGGAGCTT-3’; DD60: 5’-TGCCGAAGCTTCGACTGT-3’;
DD6: 5’-GATCAAGAGCATCGAGAA-3’;
DD7: 5’-GAYCTSAARCCVGAARAA-3’.
In order to improve the reproducibility, the improved differential display protocol was used in our study (Von der Kammer et al., 1999). Aliquots of 2 μl of the obtained cDNA each were subjected to PCR employing the corresponding one base anchor oligonucleotide along with either one of the DD (differential display) random primers, 1.5 mM MgCl₂, 0.20 mM dNTP, 1 U Taq polymerase in a 20 μl final volume. PCR conditions were performed as follows: one round at 94 °C for 1 min; 40 °C for 4 min; 72 °C for 1 min. This round was followed by 40 cycles: 94 °C for 45 s; 60 °C for 2 min; 72 °C for 1 min. One final step at 72 °C for 5 min was added to the last cycle.

2.5. Electrophoresis

PCR products were separated on 0.4 mm thick, 4% denaturing polyacrylamide sequencing gels in a temperature-regulated Bio-Rad Sequencing System (Bio-Rad, California, USA) at 50 °C. Gels were silver-stained and photographed.

2.6. Cloning, sequencing and reverse-northern blot

Bands that showed differences between reciprocal cross-fertilized kernel (Nongda3338 × Jingdong6) and its parents were excised from the gel and reamplified using the following PCR conditions: 1 min at 94 °C; 45 s at 94 °C, 2 min at 60 °C, 1 min at 72 °C, followed by 40 cycles; one final step at 72 °C for 5 min was added to the last cycle. To ensure that there is no DNA contamination in RNA samples, a negative control was prepared without reverse transcription. The wheat-differential-expression-genes fragments (WDEGFs) were ligated into pGEM-T easy vector (Promega, Madison, USA) and sequenced. Reverse-northern analysis was performed according to manufacturer’s instruction (ECL kit, Amersham, UK) with minor modifications. Each fragment was reamplified and run on a 1.0% agarose gel and transferred to a nylon membrane. The total RNA was ECL-labeled and hybridized to a Hybond N+ membrane (Amersham, UK) according to manufacturer’s recommendations.

2.7. In silico expression analysis

Statistical analysis of gene expression profiles was performed using EST data as constituents of Unigene. Similarity of gene expression was estimated using Pearson’s correlation coefficient, as described by Eisen et al. (1998). The hierarchical clustering method (Eisen et al., 1998) was applied to compare differentially expressed genes profiles among the 7 tissues. The expression profile is displayed based on the number of constituents in a Unigene (Table 1).

## 3. Results

3.1. Differential expression profiles in reciprocal cross-fertilized kernels and their parents

A total of 2859, 2831, and 2430 cDNA fragments were amplified in the kernels at 2DAP, 6DAP, and 12DAP respectively, using 24 primer combinations which include three one-base anchored primers and eight 5’ end oligonucleotide primers. The banding patterns of the differentially displayed cDNAs include quantitative difference and qualitative difference. Since quantitative differences could not be accurately examined, we only analyzed the qualitative differences which include eight categories, that is, bands observed in both hybrids but neither of the parents (BF₁nP, Fig. 1A), bands present in one parent and two hybrids but absent in the other parent (UF₁, Fig. 1B), bands occurring in only one parents but absent in two hybrids and the other parent (UPnF₁, Fig. 1C), bands detected in both parents but not in two hybrids (BPnF₁, Fig. 1D), bands displayed in only one hybrid (BoHnP, Fig. 1E), bands visualized in both parents and one hybrid (BPoH, Fig. 1F), bands revealed in one hybrid and its corresponding paternal parent (BHM, Fig. 1G), bands detected in one hybrid and its corresponding maternal parent (BHP, Fig. 1H).

While analyzed across the 3 hybrids, BF₁nP pattern accounts for 2.80%, 5.63% and 6.17% at 2DAP, 6DAP and 12DAP, respectively. UF₁ pattern accounts for 1.75%, 6.83% and 8.03% at 2DAP, 6DAP and 12DAP, respectively. UPnF₁ pattern accounts for 2.80%, 5.63% and 6.17% at 2DAP, 6DAP and 12DAP, respectively. BoHnP pattern accounts for 6.83%, 12.07% and 10.97%, BPoH pattern accounts for 3.81%, 7.13% and 6.43%, BHM pattern accounts for 10.4%, 10.63% and 9.93%, BHP pattern accounts for 5.4%, 5.83% and 6.87%, respectively.

In this study, we further analyzed that the banding patterns of the differentially displayed cDNA, named BF₁nP, UF₁, UPnF₁ and BPnF₁ are determined by genotype of hybrid, it about accounted for 19.47%, 26.78% and 35.44% at 2DAP, 6DAP and 12DAP, respectively.
12DAP, respectively. Among them, the patterns of BF1nP and BPnF1 represent the overdominance effect, it about accounted for 4.55%, 7.84% and 9.00% at 2DAP, 6DAP and 12DAP, respectively; the patterns of UPF1, and UPnF1 represent the dominance effect, it about accounted for 14.92%, 18.94% and 26.44% at 2DAP, 6DAP and 12DAP, respectively. The pattern of BoHnP and BPoH is determined by maternal cytoplasm-genotype of hybrid interaction, it about account for 10.64%, 19.20% and 17.4% at 2DAP, 6DAP and 12DAP, respectively. BHM represented female-of-origin effects and BHP represented male-of-origin effects.

3.2. Cloning, confirmation and sequencing of differentially expressed cDNAs

Seventeen cDNA fragments differentially expressed as detected in DD (differential display) between two hybrids (Nongda3159 × Jingdong6 and Jingdong6 × Nongda3159) and their parents at 6DAP were cloned and their expression patterns were confirmed by reverse-northern blot (Fig. 2). Among the differentially expressed cDNAs (Table 2), one transcript (WDEGF1) was expressed in both hybrids but neither of the parents, two transcripts (WDEGF2, WDEGF7) showed expression in only one hybrid; two transcripts (WDEGF3, WDEGF6) revealed in one hybrid and its corresponding paternal parent; one transcript (WDEGF4) visualized in both parents and one hybrid; three transcripts (WDEGF5, WDEGF8 and WDEGF9) present in one parent and two hybrids but absent in the other parent; two transcripts (WDEGF10, WDEGF12) observed in both parents and two hybrids, but the expression abundance is lower in one hybrid than those in its parents and the other hybrid; three transcripts (WDEGF11, WDEGF16 and WDEGF17) revealed in both parents and two hybrids, but the expression abundance in hybrids is similar to that in high parent; two transcripts (WDEGF13, WDEGF14) observed in both parents and hybrid, but the expression abundance higher in one parent than those in hybrids and the other parent; the expression abundance of one transcript (WDEGF15) in hybrid F1 is similar to its corresponding paternal parent.

Homology search in GenBank database showed that six transcripts (WDEGF1, WDEGF4, WDEGF8-9, WDEGF13 and WDEGF17) have no homologous hits to any known gene. Three transcripts had similarity to genes which were involved in...
the pathways of signal transduction, such as, WDEGF3 is similar to CREB-binding protein (CBP), WDEGF6 is similar to C3HC4 zinc-binding protein and WDEGF15 is similar to CaM binding protein. Four transcripts (WDEGF2, WDEGF5, WDEGF12 and WDEGF16) showed high similarity to ABC transporter ATP-binding protein, sedoheptulose-1,7-bisphosphatase, photosystem II chlorophyll a-binding protein psbB, and glucan synthase, respectively. One transcript (WDEGF10) had a high similarity to the plant NBS-LRR type resistance protein. Two transcripts (WDEGF7, WDEGF14) showed high similarity to structure maintain of chromosome protein (SMC) and RNA-directed RNA polymerase, respectively. One transcript (WDEGF11) had high similarity to gag-pol polyprotein (Table 2).

### 3.3. In silico expression profile of differentially expressed cDNAs

As of August 2005, there were 35,263 wheat Unigene clusters in NCBI database and expression profiles of the most Unigene clusters were generated based on counts of ESTs homologous to each Unigene. We found that, except for WDEGF2, 4, 6, 8, 10, 12, 13, 17, other 9 differentially expressed cDNA had a representative Unigene cluster, and the expression profiles of these Unigene clusters were already constructed. Based on these analyses, in silico expression patterns of these differentially expressed cDNAs were investigated. The number of EST homologous to each differentially expressed cDNAs ranged from 2 to 152. In silico expression analysis of the 9 differential expressed genes indicated that they are expressed in various tissue-specific patterns (Table 3). Expression profiles in different tissues were derived from each of these 9 differentially expressed cDNAs. The number of EST constituents assigned to 7 tissues was scored for each cDNAs, producing a two-way expression profile, i.e. genes versus tissues. This matrix table (Table 3) represented the primary data for further computations. Based on Pearson’s correlation coefficient for the EST constituent matrix, hierarchical clustering was constructed according to the method of Eisen et al. (1998). Based on these expression patterns, it was worthy to note that the

### Table 2

<table>
<thead>
<tr>
<th>Code</th>
<th>Name</th>
<th>DDRT expression patterns</th>
<th>Reverse-northern blot validation expression patterns</th>
<th>BLAST search E value</th>
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<tr>
<td>1</td>
<td>WDEGF1</td>
<td>BF, nP</td>
<td>BF, nP</td>
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<td>2</td>
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<td>BoHnP</td>
<td>ABC transporter ATP-binding protein 1e−106</td>
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<tr>
<td>3</td>
<td>WDEGF3</td>
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<td>BHP</td>
<td>CREB-binding protein(CBP) 2e−14</td>
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<tr>
<td>4</td>
<td>WDEGF4</td>
<td>BPoH</td>
<td>BPoH</td>
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</tr>
<tr>
<td>5</td>
<td>WDEGF5</td>
<td>UPF₁</td>
<td>UPF₁</td>
<td>Sedoheptulose-1,7-bisphosphatase 3e−77</td>
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<tr>
<td>6</td>
<td>WDEGF6</td>
<td>BHP</td>
<td>BHP</td>
<td>C3HC4 zinc-binding protein 2e−19</td>
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<td>7</td>
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<td>BoHnP</td>
<td>Structure maintain of chromosome protein (SMC) 1e−29</td>
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<tr>
<td>8</td>
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<td>UPF₁</td>
<td>No homologous sequence</td>
</tr>
<tr>
<td>9</td>
<td>WDEGF9</td>
<td>UPF₁</td>
<td>UPF₁</td>
<td>No homologous sequence</td>
</tr>
<tr>
<td>10</td>
<td>WDEGF10</td>
<td>BPoH</td>
<td>BPLH</td>
<td>NBS-LRR type resistance protein 2e−27</td>
</tr>
<tr>
<td>11</td>
<td>WDEGF11</td>
<td>UPF₁</td>
<td>HPF₁</td>
<td>Gag-pol polyprotein 1e−31</td>
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<tr>
<td>12</td>
<td>WDEGF12</td>
<td>BPoH</td>
<td>BPLH</td>
<td>Photosystem II chlorophyll a-binding protein psbB 4e−82</td>
</tr>
<tr>
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<td>WDEGF13</td>
<td>UPnF₁</td>
<td>HUP</td>
<td>No homologous sequence</td>
</tr>
<tr>
<td>14</td>
<td>WDEGF14</td>
<td>UPnF₁</td>
<td>HUP</td>
<td>RNA-directed RNA polymerase 7e−40</td>
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<tr>
<td>15</td>
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<td>BHSₚ</td>
<td>CaM binding protein 4e−41</td>
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<td>HPF₁</td>
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<tr>
<td>17</td>
<td>WDEGF17</td>
<td>UPnF₁</td>
<td>HPF₁</td>
<td>No homologous sequence</td>
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</tbody>
</table>

BPLH: bands observed in both parents and two hybrids, but the amount of expression in one hybrid is lower than its parents and the other hybrid. HPF₁: bands observed in both parents and two hybrids, but the amount of expression in hybrids is similar to high parent. HUP: bands observed in both parents and hybrid, but the amount of expression in one parent is higher than hybrids and the other parent. BHSₚ: the amount of expression in hybrid F₁ is similar to its corresponding paternal parent.

### Table 3

<table>
<thead>
<tr>
<th>Name</th>
<th>Unigene cluster</th>
<th>Silico expression profile (the number of EST per million)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Crown</td>
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<tr>
<td>WDEGF1</td>
<td>Ta.38581</td>
<td>0</td>
</tr>
<tr>
<td>WDEGF3</td>
<td>Ta.1393</td>
<td>0</td>
</tr>
<tr>
<td>WDEGF5</td>
<td>Ta.1988</td>
<td>0</td>
</tr>
<tr>
<td>WDEGF7</td>
<td>Ta.7619</td>
<td>135</td>
</tr>
<tr>
<td>WDEGF9</td>
<td>Ta.5064</td>
<td>0</td>
</tr>
<tr>
<td>WDEGF11</td>
<td>Ta.33187</td>
<td>68</td>
</tr>
<tr>
<td>WDEGF14</td>
<td>Ta.40242</td>
<td>0</td>
</tr>
<tr>
<td>WDEGF15</td>
<td>Ta.9486</td>
<td>0</td>
</tr>
<tr>
<td>WDEGF16</td>
<td>Ta.32366</td>
<td>0</td>
</tr>
</tbody>
</table>

expression of 7 differentially expressed fragments (WDEGF1, 7, 9, 11, 14, 15, 16) showed significant correlation coefficients ($r=0.87$, $P<0.05$), and they were clustered into one group. These results might imply that the 7 differentially expressed genes were regulated by coordinate gene expression (Fig. 3).

4. Discussion

Although most of the major discernible morphogenetic events in plant occur after germination, the overall architecture pattern of the mature plant is established during embryogenesis (Thomas, 1993). Several studies have shown that the cross-fertilization advantage was expressed during the first stage of embryo development and grain filling. In fact, plant embryogenesis is a very complex and highly organized process, which is developmentally regulated by a large number of genes and it was reasonable that the genetic regulation during the seed development should be the primary determinant for heterosis. In our previous study, the differential gene expression between wheat hybrids and their parental inbreds in leaves were analyzed via DDRT-PCR and the results showed that about 11%, 30% and 27% of total displayed cDNAs were polymorphic at the male-of-origin and female-of-origin, which appears that kernel development is strongly influenced by its own genotype, is partially determined by the maternal genotype. Based on the investigation of phenotype evolution, it is suggested that phenotype evolution often proceeds through the tempo-spatial changes in gene expression (Doebley and Lukens, 1998). The phenomenon of heterosis observed in hybrid $F_1$ can also be considered as a type of phenotype evolution, which occurs in hybrids produced through hybridization of two parents. In this study, we found that the differentially expressed genes between reciprocal cross-fertilized kernels include those that are involved in signal transduction, metabolism, and so on. WDEGF3, WDEGF6 and WDEGF15 had high similarity to CREB-binding protein (CBP), C3HC4 zinc-binding protein and CaM binding protein, respectively. CREB-binding protein, CBP, which activates the cAMP dependent signaling pathway, a pathway used by many hormones and neurotransmitters to regulate cellular activities and gene expression, CBP and its homologue p300 were found to be universal coactivators of many classes of transcription factors, CBP and p300 are now known to acetylate histones, a process linked to the chromatin remodeling and gene activation, signifying the roles of CBP and p300 in gene transcription (Kwok, 1994). The plant CaM binding protein was involved in $Ca^{2+}$/CaM-mediated signaling pathways related to morphogenesis, cell division, cell elongation, ion transport, gene regulation, cytoskeletal organization, cytoplasmic streaming, pollen function, and stress tolerance (Reddy et al., 2002). Although we still cannot speculate how the changes of signaling transduction pathways in hybrid will affect the heterosis, the differentially expressed genes involved in multiple signaling pathways might play certain important roles in the heterosis formation. Three transcripts (WDEGF5, WDEGF12 and WDEGF16) showed high similarity to gene involved in metabolism, sedoheptulose-1,7-bisphosphatase, photosystem II chlorophyll a-binding protein psbB, and glucon synthase, respectively. Miyagawa et al. (2001) reported that overexpression of sedoheptulose-1,7-bisphosphatase in tobacco enhances photosynthesis and growth, Vermaas (1998) indicated that the PsbB protein is an absolute requirement for photoautotrophic growth, and we also found a sedoheptulose-1,7-bisphosphatase-like gene, psbB-like gene, were differentially expressed at the two hybrids and its parents and this indicated that the cloned two genes might enhance photosynthesis of hybrids, and produce heterosis.

WDEGF7 had high similarity to Structure Maintain of Chromosome protein (SMC), SMCs were initially identified in *S. cerevisiae* and have been identified in all eukaryotic organisms examined to date and fall into six clearly discernible subfamilies (SMC1-4, Rad18, and Rad18 related). All SMC proteins are large polypeptides that share partial sequence conservation and similar structural organization (Cobbe and Heck, 2000). The N-terminal end contains a putative Walker A motif (ATP binding domain), and at the C-terminal end is located a characteristic “DA” box (Walker B motif) involved in ATP hydrolysis (Saitoh et al., 1994). These domains are separated by a coiled-coil region, interrupted by a central globular hinge region. A complex containing a heterodimer of SMC2 and SMC4 has been shown to function in chromosome condensation (Hirano and Mitchison, 1994; Saka et al., 1994; Strunnikov et al., 1995; Sutani et al., 1999), while an analogous complex containing an SMC1/SMC3 heterodimer plays a role in sister chromatid cohesion (Guacci et al., 1997; Losada et al., 1998;
Michaelis et al., 1997). In this paper, a gene encoding an SMC-like protein is differentially expressed in hybrid and its parents and this indicated that proteins involved in the chromosome stabilization might be benefit to heterosis.

In this paper, we analyzed gene expression in one important stage of the wheat growth and development and this stage might not the sole crucial stage for heterosis in all the traits. Analysis of gene expression in other crucial stage should be conducted. Further studies are needed to isolate the full length cDNAs of those cloned genes which are differentially expressed in hybrid and their parents, and to characterize their physiological functions leading to the heterosis. As well as, it is a critical task to clone more differentially expressed genes and analyze their transcriptional levels in the more selected hybrids, and find a relationship between the levels of gene expression changes and heterosis effect.

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