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Identification of differentially expressed genes in leaf and root between wheat hybrid and its parental inbreds using PCR-based cDNA subtraction

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Abstract

Heterosis was defined as the advantage of hybrid performance over its parents in terms of growth and productivity. Previous studies showed that differential gene expression between hybrids and their parents is responsible for the heterosis; however, information on systematic identification and characterization of the differentially expressed genes are limited. In this study, an interspecific hybrid between common wheat (*Triticum aestivum*. L., $2n = 6x = 42$, AABBDD) line 3338 and spelt (*Triticum spelta* L. $2n = 6x = 42$, AABBDD) line 2463 was found to be highly heterotic in both aerial growth and root related traits, and was then used for expression assay. A modified suppression subtractive hybridization (SSH) was used to generate four subtracted cDNA libraries, and 748 nonreduandant cDNAs were obtained, among which 465 had high sequence similarity to the GenBank entries and represent diverse of functional categories, such as metabolism, cell growth and maintenance, signal transduction, photosynthesis, response to stress, transcription regulation and others. The expression patterns of 68.2% SSH-derived cDNAs were confirmed by reverse Northern blot, and semi-quantitative RT-PCR exhibited the similar results (72.2%). And it was concluded that the genes differentially expressed between hybrids and their parents involved in diverse physiological process pathway, which might be responsible for the observed heterosis.

Abbreviations: BPH, best-parent heterosis; GO, gene ontology; LRL, longest root length; MPH, mid-parent heterosis; RAD, root average diameter; RDW, root dry weight; RSA, root surface area; RSR, root/shoot ratio; RTN, root tips number; RT-PCR, reverse transcription polymerase chain reaction; RV, root volume; SSH, suppression subtractive hybridization; TL, taproot length; TN, taproot number; TRL, total root length

Introduction

Hybrid cultivars have been used commercially in many crops and have made significant contribution to the world food supply (Duvick, 1997). However, molecular mechanism of heterosis remains to be revealed. It has been suggested that molecular foundation of phenotypic changes could

reside in the variability of genome expression (Doebley and Lukens, 1998). Although all the genes in hybrid F_1 are derived from its parental inbreds, hybrid performance is quite different from its parental inbreds. Therefore, it is reasonable to speculate that differential gene expression between hybrids and their parents should be responsible for the observed heterosis (Ni *et al*., 2000; Wu *et al*., 2003; Sun *et al*., 2004). Previous studies detected significant difference in mRNA quantity and expression patterns between hybrids and their parental inbreds (Sun *et al*., 1999). Further analysis indicated that differential gene expression patterns in leaf tissue in rice and wheat were correlated with heterosis (Xiong *et al*., 1998; Sun *et al*., 2004). However, changes on the level of mRNA do not necessarily indicate changes on the protein level and/or in the hybrid phenotype, further studies are needed to isolate differentially expressed genes, and investigate their functional relations to heterosis.

Recently, attempts have been made to clone and characterize differentially expressed cDNAs between hybrid and its parents. Ni *et al*. (2000) cloned a cDNA encoding for a novel RNA-binding protein that was specifically expressed in F_1 but not in the parents and discussed its possible role in wheat heterosis. Wu *et al*. (2003) reported the cloning of 24 differentially expressed leaf cDNAs between wheat hybrids and their parents. To date, these analyses only relied on a small sampling of gene expression rather than a comprehensive examination of genome-wide expression patterns (Birchler *et al*., 2003). A larger sampling could provide more insight into the spectrum of genes of which the expression altered in hybrids and in which direction the changes occurs. Thus, a more complete comparison of gene expression profiles should be meaningful, which might be able to formulate the molecular model of heterosis. A modified 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 wheat hybrid and its parents both in leaves and roots harvested at tillering stage. Totally 926 differentially expressed cDNAs were sequenced and used for BLAST searches of the NCBI database, and the expression profiles of roots cDNAs were determined by reverse Northern blot. And the expression patterns of 18 randomly selected cDNAs from roots and leaves were analyzed by semi-quantitative reverse transcription polymerase chain reaction (RT-PCR). To test whether differentially expressed cDNA fragments truly represent the putative genes and provide a basis for further analysis of their function, complete open reading frames (ORF) of 10 selected cDNA fragments were assembled using *in silico* cloning and their expression patterns were validated by RT-PCR.

Material and methods

Plant materials

One highly heterotic interspecific hybrid 3338/2463 and its female parent 3338 (*Triticum aestivum* L., $2n = 6x = 42$, AABBDD), male parent 2463 (*Triticum spelta* L. $2n = 6x = 42$, AABBDD) were used for this study. For measurement of the heterosis in vigorous tillering stage, pre-germinated seeds of hybrid and its parents were cultivated with 8 replications in two types of planting medium, nutrient solution (Broughton and Dilkworth, 1971) that was changed every 4 days and vermiculite that was watered by nutrient solution. The seedlings were grown in a growth chamber at a relative humidity of 75% and 26/20 °C day and night temperature with light supplementation to reach at least 3000 lx. Roots from 8-week tillering seedlings in vermiculite and 4-week seedlings in nutrient solution were used to measure root traits and estimate heterosis. Meantime, for suppression subtractive hybridization, the leaves and roots of the hybrid and its parents from four seedlings with 4 tillers cultivated in vermiculate were harvested and frozen in liquid nitrogen and stored at −80 °C before use. The tissues of hybrid and its parents such as roots and leaves in trefoil stage, leaves and shoots in jointing

stage, spikes and internode below spike in booting stage were collected for RT-PCR analysis. In addition, leaves, shoots and roots of inbred line 3338 seedling in trefoil stage were also collected for tissue specific analysis, and the spikes in different developmental stages such as booting, heading and anthesis stage were used to analyze the temporal expression profiles.

Root heterosis measurement

Root architectural traits were analyzed following a slightly modified procedure of Yabba and Foster (2003) and Frahm *et al*. (2003) using the software WinRHIZOTM (WinRHIZO, Regents Instruments Inc., 2001, Quebec, Canada). Individual root systems were transferred to a 30 cm × 20 cm plexi-glass plate for scanning after floated in clear water and carefully dispersed into individual lateral and secondary roots with forceps as far as practicable to prevent overlapping (Harris and Campbell, 1989) (Figure 1B). Although root systems develop a three-dimensional form in the soil, roots were measured in two dimensions in this study. The aerial parts and root system were drying in a vacuum oven at 80 °C for 72 h for dry weight determination and root/shoot ratio calculation. The longest root length (cm) and taproot number were measured manually. The mid-parent heterosis (MPH) and best-parent heterosis (BPH) were calculated using following formula: MPH = (mean F_1 – mean P)/mean P in %; BPH = (mean F_1 – mean best P)/mean best P in %. Statistical analysis of the differences in root traits was performed using *t*-test.

RNA extraction

Total RNA was isolated from each sample by using the RNeasy kit (Sangon, Shanghai, China) according to the manufacturer's instructions. Poly(A) RNA was purified from total RNA using the PolyATract mRNA isolation system (Promega, Madison, WI, USA).

Suppression subtractive hybridization and SSH cDNA library construction

Suppression subtractive hybridization was performed by using PCR-based cDNA subtraction kit (Clontech) according to the manufacturer's protocol. Four subtracted cDNA libraries, designated FSL (forward subtractive library of leaves), RSL (reverse subtractive library of leaves), FSR (forward subtractive library of roots), and RSR (reverse subtractive library of roots), were constructed with enriched gene expressed specifically or at higher level in hybrid leaves, parent leaves, hybrid roots and parent roots, respectively (Table 1). Equal amount (1 μg) poly(A) RNA of two parents were mixed, reverse transcripted into cDNAs and used as tester/driver for suppression subtractive hybridization with cDNAs derived from 2 μg hybrid poly(A) mRNA. The cDNAs were digested with *RsaI* and ligated to different adaptors (Adaptor1: 5′-CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCGGGCAGGT-3′; Adaptor2R: 5′-CTAATACGACTCACTATAGGGCAGCGTGGTCGCGGCCGAGGT-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 as the primary 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.

Table 1. Construction of four subtractive libraries.

Subtractive library	Tester	Driver	Enriched genes in the library
FSL	Hybrid leaf cDNAs	Parents leaf cDNAs	Hybrid leaf specific expressed
RSL	Parents leaf cDNAs[a]	Hybrid leaf cDNAs	Parents leaf specific expressed
FSR	Hybrid root cDNAs	Parents root cDNAs	Hybrid root specific expressed
RSR	Parents root cDNAs[b]	Hybrid root cDNAs	Parents root specific expressed

[a] Parents leaf cDNAs were derived from the mixture of two parents mRNA from leaf.
[b] Parents root cDNAs were derived from the mixture of two parents mRNA from root.

Sequencing and analysis of the subtracted cDNA

White colonies from the 4 subtractive cDNA libraries were picked up and amplified by PCR using SP6 and T7 primers. The PCR products were analyzed by running on 3.5% native polyacrylamide gels (*N*,*N*′-methylene-bis-acrylamide:acrylamide = 19:1) to confirm the positive colonies. Nine hundred and twenty-six differentially expressed cDNA clones were selected for sequencing and the sequences were used for a BLAST search of the NCBI (http://www.ncbi.nlm.nih.gov) protein database using BLASTX with E-value thresholds e^{-10}. The putative physiological functions of sequences were classified according to the GeneOntology (http://www.geneontology.org).

Reverse-Northern blot analysis

Nonredundant cDNA colonies were printed on three identical Hybond N+ membranes (Amersham, UK). The inclusion of housekeeping genes such as β-actin with two duplications on the array served to determine hybridization efficiency and to enable normalization between blots. Template cDNA were generated by reverse transcription of total RNA of hybrid and its parents using M-MLV reverse transcriptase (Promega, Madison, USA) according to the manufacturer's instructions. The reaction mixture contains 50 μCi 32P-dCTP. The cDNA products were added to reverse-Northern blot buffer. The three membranes were prehybridized for 16–18 h at 68 °C and then hybridized overnight at 68 °C to labeled cDNAs of hybrid and its two parents, respectively. After washing two times with 2 × SSC, 1% SDS at 68 °C for 15 min, the membranes were exposed to X-ray films for autograph at −80 °C. Images were acquired by scanning the membranes with a FluorChem™ (Alpha innotech). Signal intensity of spot was analyzed using FluorChem™ 5500 software. Statistical analysis of the differences in signal intensity was performed using *t*-test and LSD method.

Semi-quantitative RT-PCR analysis

Two microgram total RNA of each sample was used for first-strand cDNA synthesis in 20 μl reactions containing 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 50 μM dNTPs, 200 U M-MLV reverse transcriptase (Promega, Madison, USA) and 50 pmol oligonucleotides T15. Reverse transcription was performed at 37 °C for 60 min with a final denaturation at 95 °C for 5 min. Gene-specific RT-PCR primers for the 18 differentially expressed cDNA fragments were designed according to the cDNA sequences (Table S1).

Three RT-PCR replications were conducted using independently isolated total RNAs with the following thermal cycling parameters: 94 °C for 30 s, 68 °C for 1 min, and 72 °C for 1 min. Various numbers of PCR cycles were tested to ensure that the reactions had not reached the plateau. A 350 bp β-actin gene fragment was amplified as a positive control using the primer pair 5′-CAGCAACTGGGATGATATGG-3′ and 5′-ATTTCGCTTTCAGCAGTGGT-3′. The RT-PCR products were sequenced to verify the specialty of PCR amplification. For quantification, the intensity of the PCR bands was estimated with FluorChem™ 5500 software.

In silico cloning of complete open reading frames (ORF)

Ten differentially expressed cDNAs were selected for Blast search of the NCBI EST database. The overlapping ESTs were assembled into contigs to obtain the open reading frames (ORFs). Specific primers were designed for each ORFs (TableS2) and the PCR products were sequenced.

Results

Heterosis of the interspecific hybrid in the vigorous tillering stage

Previous field test demonstrated that the hybrid 3338 × 2463 yielded 20% more than the higher-yielding parent in terms of grain yield per plant (our unpublished data). However, as Pickett (1993) points out, many other characteristics related to yield performance might also exhibit heterosis. In this study, we found that the hybrid 3338 × 2463 had larger aerial parts and roots system than the parental lines at tillering stage both in nutrient solution and vermiculite (Figure 1A). The MPH of aerial parts and roots biomass can reach 23.3%, 35.3% in nutrient solution and 39.9%, 75.3% in vermiculite,

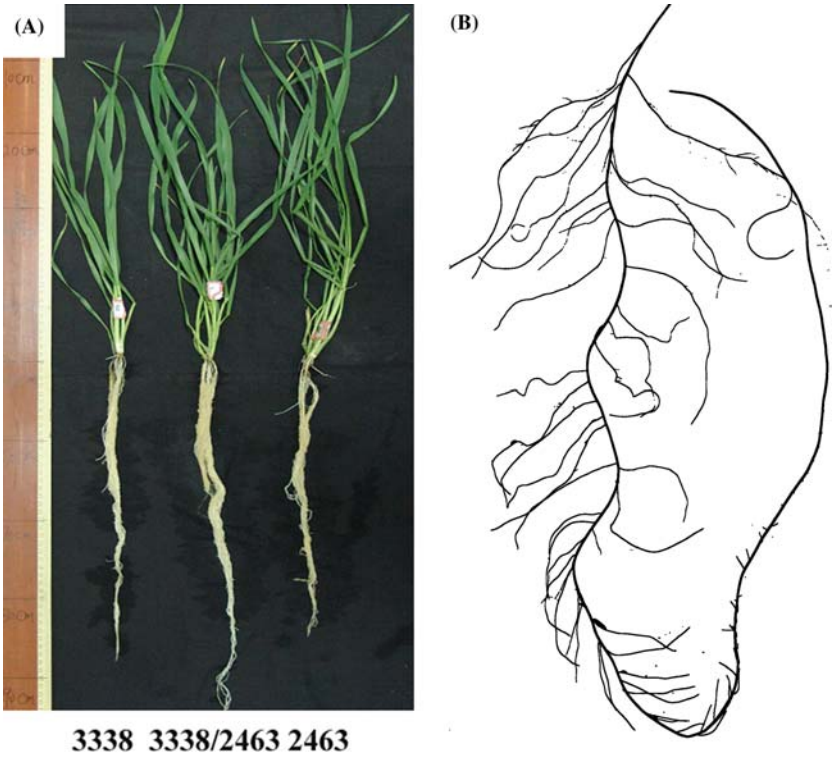

Figure 1. (A) Developmental phenotypes comparisons of wheat hybrid 3338/2463 and two parents 3338 and 2463 in tillering stage. Roots and arieal parts were harvested for suppression substractive hybridization. (B) Scanning picture of roots for root traits analysis. Roots of hybrid and its parents were scanned and analyzed using the scanner and software WinRHIZOTM (WinRHIZO, Regents Instruments Inc., 2001, Quebec, Canada). Individual root systems were transferred to a 30 cm × 20 cm plexi-glass plate for scanning after floated in clear water and carefully dispersed into individual lateral and secondary roots with forceps as far as practicable to prevent overlapping.

respectively, indicating that root heterosis is much higher than that of aerial parts (Table 2). Since roots biomass exhibited more obvious heterosis, 10 root characters in two planting medium were measured including total root length (TRL), root surface area (RSA), root average diameter (RAD), root tips number (RTN), longest root length (LRL), taproot

Table 2. Mid-parent heterosis [MPH = (mean F_1 − mean *P*)/mean *P* in %] and best-parent heterosis [BPH = (mean F_1 − mean best *P*)/mean best *P* in %] between 3338 and 2463 of roots in the vigorous tillering stage.

Heterosis[$]	Trait[#]										
	TRL	RSA	RAD	RV	RTN	RDW	ADW	LRL	TN	TL	RSR
MPH (NS)	28.54**	27.52*	0.53	24.68*	20.02*	35.32**	23.33*	14.72	12.46	12.07	9.45
BPH (NS)	23.66*	22.48*	−8.34	9.40	8.42	25.52*	11.96	12.55	9.87	17.67	13.70
MPH (VM)	104.08*	96.64**	−8.11	80.72*	101.48	75.30**	39.94**	6.71	25.71	–	22.16
BPH (VM)	70.89	66.21*	−15.00	60.24	74.59	53.97*	16.24*	11.32	22.22	–	15.43

Hybrid and its two parents were planted in nutrient solution and vermiculite with 8 replications. Statistical analysis of the differences in root traits was performed using *t*-tests.

[#] TRL: total root length; RSA: root surface area, RAD: root average diameter, RTN: root tips number, LRL: longest root length, TN: taproot number, TL: taproot length, RV: root volume, RDW: root dry weight, ADW: aerial dry weight and RSR: root/shoot ratio.

[$] NS represents the nutrient solution and VM represents the vermiculite medium.

** Significant difference with *P*-value < 0.01.

* Significant difference with *P*-value < 0.05.

number (TN), taproot length (TL), root volume (RV), root dry weight (RDW) and root/shoot ratio (RSR). A quantification of mid-parent and best parent heterosis and the results of statistical significance tests were shown in Table 2. We observed significant MPH ($P < 0.05$) for four traits (TRL, RSA, RV and RDW) both in nutrient solution and vermiculite. Furthermore, we also observed the significant MPH for total root number (TRN) in nutrient solution. However, no significant difference between hybrid and its parents was observed for traits RSR, LRL, TN and TL, indicating that F_1 hybrid showed higher lateral roots density and length than its parents (Table 2). The heterotic performance of leaf and root at the vigorous tillering in two planting medium suggested that leaf and root can be used as ideal materials to investigate molecular basis of wheat heterosis.

Functional classification of suppression subtractive hybridization identified nonredundant ESTs that are differentially expressed between hybrid and parent

From the four subtraction libraries, 926 differentially expressed cDNA colonies were selected for sequencing after PCR screening of 2500 colonies. The cDNA insert size ranged from ~100

Table 3. The number of differentially expressed cDNAs identified from 4 subtractive libraries.

Subtractive library[a]	Total clones	Nonredundant clones	Best match[b]	Low score[c]	No hit[d]
FSL	262	162 (62.1%)	93	52	17
RSL	151	124 (82.1%)	93	25	6
FSR	291	266 (87.5%)	185	69	12
RSR	222	196 (87.9%)	94	61	41
Total	926	748	465	207	76

[a] FSL, RSL, FSR, RSR denotes forward subtractive library of leaves, reverse subtractive library of leaves, forward subtractive library of roots, and reverse subtractive library of roots, respectively.
[b] Best match denotes the sequences that has high sequence similarity (BLASTX expectation values [*E*] of $<10^{-10}$) to the GenBank entries.
[c] Low score denotes the sequences that has low sequence similarity (BLASTX expectation values [*E*] of $>10^{-10}$) to any database entries.
[d] No hit indicates the sequences that have no homologies with any genes in the database.

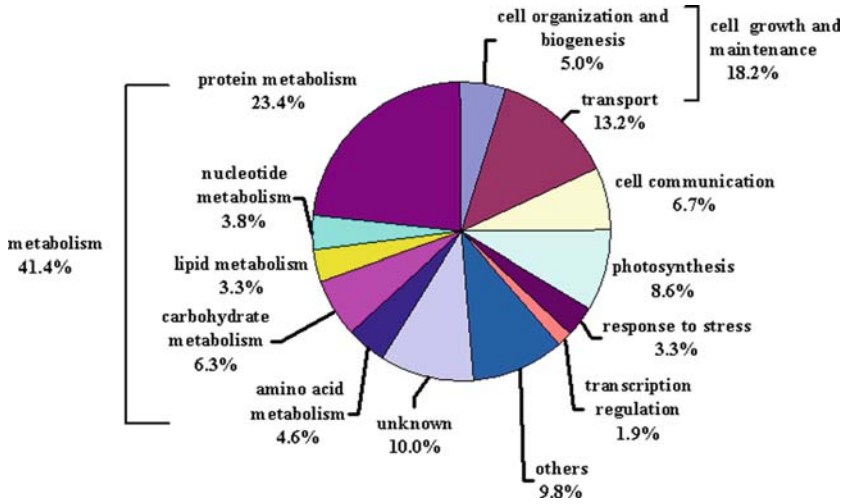

Figure 2. The functional classification of 465 cDNAs with high sequence similarity to the GenBank entries using Gene Ontology (GO) classification scheme (Gene Ontology Consortium, http://www.geneontology.org). ESTs with GO assignments are collated and plotted as a percentage of GO-annotated ESTs. Some classifications were pooled to reduce the member of classes. Protein, nucleotide, lipid, carbohydrate and amino acid metabolism were polled in a general name metabolism. Category 'cell organization and biogenesis' and 'transport' belongs to cell growth and maintenance.

to $\sim$ 800 bp, mostly between 300 and 500 bp. Basic Local Alignment Search Tool (BLAST) (Altschul *et al*., 1997) search indicated that 748 (80.8%) cDNAs were nonredundant, and cDNAs from FSR, RSR, FSL and RSL subtracted libraries were 266, 196, 162 and 124, respectively (Table 3). These 748 nonredundant cDNAs could be classified into three groups based on the BLAST search results. The first group consists of 465 cDNAs with high similarity (BLASTX expectation values [*E*] of $< e^{-10}$) to database entries, suggesting that they are either the same gene or belong to the same gene family. However, 48 (10.0%) cDNAs of this group show high similarity to unknown or hypothetical proteins. The second group, comprising 207 cDNAs, has low sequence similarity (BLASTX expectation values [*E*] of $> e^{-10}$) to any database entries. The remaining 76 cDNAs have no homologies with any genes in the database, which might represent either previously uncharacterized sequences or fragments that are too short to reveal any significant identity.

The 465 cDNAs with high sequence similarity to the GenBank entries were selected for functional classification using Gene Ontology (GO) classification scheme (Gene Ontology Consortium, http://www.geneontology.org). These 465 cDNAs represent a large range of functional categories, which include metabolism, cell growth and maintenance, signal transduction, photosynthesis, response to stress, transcription regulation and others (Figure 2). Further classification and comparison of cDNAs from the four different subtractive libraries were listed in Figure 3. Except for photosynthesis category, which is unique to the leaf libraries, the number of cDNAs identified from each library did not show significant difference for all categories. A complete list of the 465 differentially expressed cDNA clones was provided as supplementary tables (Table S3–S13).

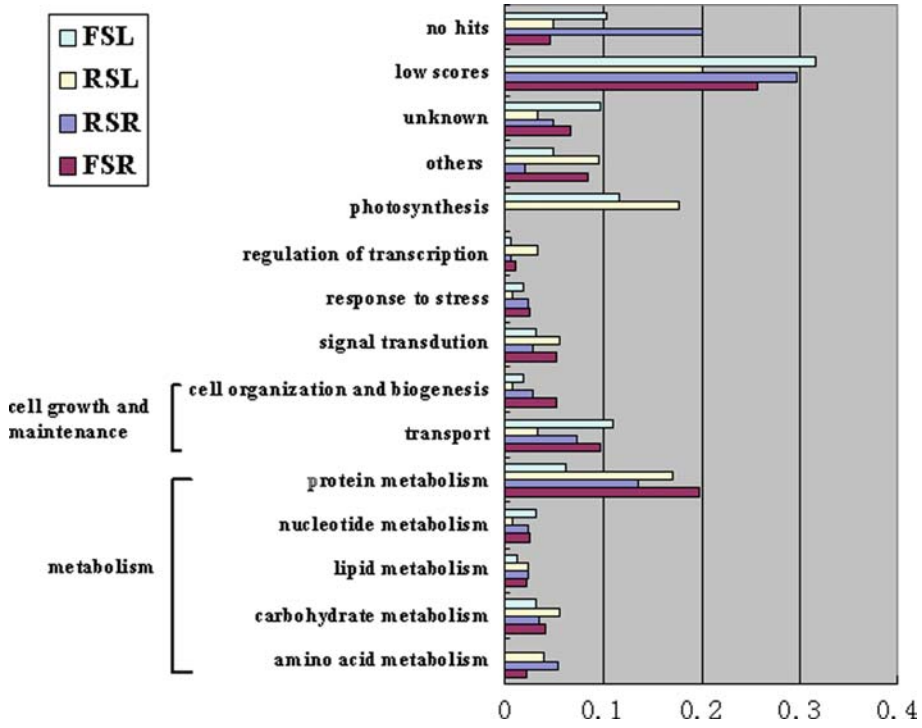

Figure 3. Classification of differentially expressed genes between hybrid and its parents in seedling leaves and roots. FSL, RSL, RSR, FSR represented ESTs from forward subtractive library of leaves, reverse subtractive library of leaves, reverse subtractive library of roots and forward subtractive library of roots, respectively. These four libraries enriched genes expressed specifically or at higher level in hybrid leaves, parent leaves, parent roots and hybrid roots, respectively. Low score represented the ESTs with low sequence similarity (BLASTX expectation values [*E*] of $> e^{-10}$) to any database entries. No hits denoted cDNAs that have no homologies with any genes in the database. Protein, nucleotide, lipid, carbohydrate and amino acid metabolism were polled in a general name metabolism. Category 'cell organization and biogenesis' and 'transport' belongs to cell growth and maintenance.

High portion of differentially expressed cDNAs (41.4%) of the 465 are involved in diverse metabolism pathways, and the largest subset of cDNAs (23.4%) in this category related to protein synthesis and degradation pathways, followed by carbohydrate metabolism (6.3%), amino acid metabolism (4.6%), lipid metabolism (3.3%) and nucleotide metabolism (3.8%) (Figure 2).

Forty-four cDNAs corresponding to thirty-five ribosomal proteins, which are essential for protein biosynthesis, expressed differentially between hybrid and its parents. Among them, 22 were from forward libraries. Three translation initiation factors (TIF) were identified from the FSR library, indicating their higher expression level in hybrid roots. Five cDNAs representing three translation elongation factors (EF-1α, eEF-1γ or eEF-2) were obtained from the reverse subtractive library. Several cDNAs related to protein degradation pathway were found differentially expressed, such as 20s proteasome β4, β5, β7, 26S proteasome AAA-ATPase subunit RPT3 and 26S proteasome regulatory subunit identified from the reverse libraries, and 20s proteasome β2, αF subunit and 26S proteasome regulatory particle non-ATPase subunit RPN12 identified from the forward libraries.

Thirty differentially expressed cDNAs were found to be involved in carbohydrate metabolism and nine of them encoded key enzymes for citric acid cycles and glycolysis pathway. We found 15 differentially expressed cDNAs involved in lipids metabolism, most of which corresponded to the fatty acids metabolism. Two cDNAs encoding acety-CoA carboxylase and b-keto acyl reductase were identified from the roots forward library, and both of them play an important role in fatty acid biosynthesis. Other fatty acid metabolism-related genes such as enoyl-CoA-hydratase, and acetoacetyl CoA thiolase, were also derived from roots forward library.

Eighteen percent (87 cDNAs) of the differentially expressed cDNAs were founded to be involved in the cell growth and maintenance. Among them, 67 cDNAs were found to participate in the direct movement of substance (such as macromolecules, small molecules), which could be either carrier or channel proteins. Eleven carrier protein cDNAs were identified only from the root libraries and nine of them were from the FSR, indicating a higher transmembrane ability in hybrid roots for ion, nucleotides, amyloses and amino acids transportation. Among the 13 channel proteins identified, 9 were from the forward libraries and 4 from the reverse libraries.

About 1.9% (9 cDNAs) of the differentially expressed cDNAs were found to be involved in transcriptional regulations. Histone deacetylase, catalyzing the modification of histone, was identified from root forward subtraction library. Nine transcription factors, such as APETALA2-like protein, zinc finger proteins, KOW domain-containing transcription factor family, basic leucine zipper protein, GAMYB-binding protein, and histone deacetylase, were found differentially expressed between hybrid and its parent.

About 8.6% (41 cDNAs) of cDNAs identified from the leaves libraries were related to all the pathways of photosynthesis. Several members of chlorophyll *a*/*b*-binding protein for light absorption and five cDNAs for the photochemical reaction centers complex were also differentially expressed. Ten nonredundant cDNAs encoding RuBisCo, the key enzyme in photosynthesis and photorespiration, were identified from the reverse and forward libraries, however nucleotide sequences difference was observed for the cDNAs derived from the two libraries.

In addition, 16 stress response cDNAs, such as peroxidases, heat shock related proteins, hairpin-induced protein, cold-regulated proteins, rust resistance proteins, jasmonate-induced proteins, grpE like protein, chaperonin, wound-responsive protein and copper chaperone, were also found expressed differentially between hybrid and its parents.

Confirmation of SSH expression patterns by reverse-Northern blot and semi-quantitative RT-PCR analysis

PCR-selected cDNA subtraction is a powerful tool for identifying differentially expressed genes. However, subtractive PCR products might contain cDNAs that are not truly differentially expressed between hybrid and its parents. In our study, poly(A) RNA of two parents was equally mixed and used as driver/tester for PCR-based cDNA subtraction. The forward libraries (FSL and FSR), not only enriched genes up-regulated in hybrid, but also contained genes with equal expression level to the highly expressed parent. Similarly, the

reverse libraries (RSL and RSR) consist of genes that are down-regulated in hybrid or equally expressed to the lowly expressed parent. To further test the reliability of SSH and compare the expression pattern of differentially expressed cDNAs between hybrid and its parents, reverse-Northern blot analysis was conduced for a subset of 443 nonredunant cDNAs obtained from the root libraries, among which 248 were from the FSR, and the other 195 were from the RSR. Reverse-Northern blotting indicated that, out of 443 cDNAs tested, 316 were differentially expressed between hybrid and its parents, 109 were expressed equally between hybrid and its two parents and 18 had no hybridization signals, resulting a 71.1% efficiency for characterizing differentially expressed genes. The differential expression was observed mostly in quantitative level, which can be clustered into four categories (Figure 4): (i) up-regulated in hybrid (URH), expression in hybrid is higher than in both female and male parents; (ii) down-regulated in hybrid (DRH), expression in hybrid is lower than in its parents; (iii) high-dominant in hybrid (HDH), expression in hybrid is equal to the highly-expressed parent; and (iv) low-dominant in hybrid (LDH), expression in hybrid is equal to the lowly-expressed parent. Among the 443 cDNAs identified by SSH, 70 (15.80%) and 79 (17.83%) of cDNAs from the forward subtractive libraries showed URH and HDH expression pattern, and among them 87 (19.64%) represented the signifi-

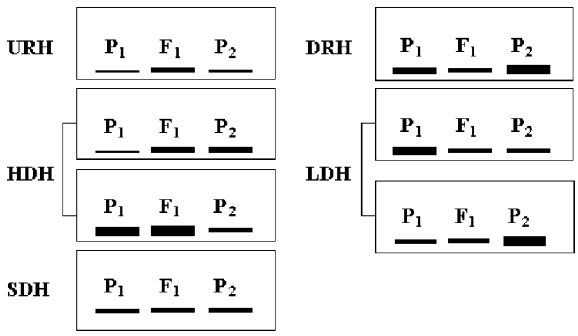

Figure 4. The analog picture represents the expression patterns between hybrid and its parents in RT-PCR and in reverse-Northern blot. URH, DRH, HDH, and LDH represent the expression pattern of up-regulated in hybrid (the expression in hybrid was higher than both female and male parents), down-regulated in hybrid (the expression in hybrid was lower than its parents), high-dominant in hybrid (gene expression in hybrid was equal to the highly-expressed parent), low-dominant in hybrid (gene expression in hybrid was equal to the lowly-expressed parent) In addition, the gene expression level between hybrid and the two parents was same (SDH).

Table 4. Reverse-Northern blot confirmation of differentially expressed cDNA fragments of roots.

Expression pattern[a]		FSR[b]		RSR[c]	
		Number (%)	Number with significant difference* (%)	Number (%)	Number with significant difference* (%)
No signal		12 (2.71%)	–	6 (1.35%)	–
URH		70 (15.80%)	30 (6.77%)	1 (0.23%)	–
DRH		7 (1.58%)	–	82 (18.51%)	35(7.90%)
HDH	FR	30 (6.77%)	25 (5.64%)	1 (0.23%)	–
	MR	49 (11.06%)	32 (7.22%)	1 (0.23%)	–
LDH	FR	2 (0.45%)	–	34 (7.67%)	20 (4.51%)
	MR	2 (0.45%)	–	37 (8.35%)	26 (5.87%)
SDH		76 (17.16%)	–	33 (7.45%)	–
Total		248 (55.98%)	–	195 (44.02%)	–

[a] URH represents that gene expression in hybrid was higher than both female and male parents; DRH represents that gene expression in hybrid was lower than both female and male parents; HDH represents that gene expression in hybrid was equal to the highly expressed parent; LDH represents that gene expression in hybrid was equal to the lowly expressed parent; SDH represent gene expression level between hybrid and the two parents was same.
FR represent that gene expression in hybrid is preferred to female parent.
MR represent that gene expression in hybrid is preferred to male parent.
[b] FSR indicate the forward subtractive library of roots.
[c] RSR indicate the reverse subtractive library of roots.
* Significant difference with P-value < 0.05.

cant differences between hybrid and its parents ($P < 0.05$). Moreover, 82 (18.51%) and 71 (16.03) cDNAs from the reverse subtractive library performed DRH and LDH expression pattern, and 81 (18.28%) of them showed significant differences between hybrid and its parents ($P < 0.05$). These results suggested that out of 443 cDNAs, 68.16% differentially expressed cDNAs identified by SSH could be validated by reverse-Northern analysis, and 168 (37.9%) showed significant expression differences between hybrid and its parents (Table 4). Semi-quantitative RT-PCR was further applied to validate the expression pattern of 18 selected cDNAs with putative functions involved in a diverse set of biological pathways (Table 5). Among them, four (*Ta*PRBP, *Ta*RK, *Ta*STPK and *Ta*G6PTP) derived from the FSR, five (*Ta*ADP, *Ta*TEF, *Ta*CDPK, *Ta*SRP and *Ta*RPT3) from RSR, six (*Ta*FTSH, *Ta*TIF, *Ta*GSH, *Ta*EIL, *Ta*FRLT and *Ta*PDI3P) from FSL and three (*Ta*SS, *Ta*TRXM and *Ta*MTH) from RSL, respectively. RT-PCR results grouped the 18 cDNAs into five expression patterns: URH, DRH, HDH and LDH as detected by reverse-Northern hybridization, and SDH which is equally expressed in hybrid and both parents. Seven out of 9 cDNAs (*Ta*PRBP, *Ta*RK, *Ta*STPK, *Ta*ADP, *Ta*TEF, *Ta*CDPK, and *Ta*RPT3) from the root subtracted libraries showed same differential expression pattern in roots as SSH and reverse-Northern results, and five of them showed significant difference between hybrid and its parents

Table 5. Eighteen differentially expressed cDNA fragments validated by RT-PCR.

Fragment name	SSH library[a]	Reverse-Northern pattern[b]	RT-PCR pattern in root[b]	RT-PCR pattern in leaf[b]	Sequence similarity	Accession number
*Ta*PRBP	FSR	HDH	HDH*	URH*	Barly putative pumilio/Mpt5 family RNA-binding protein	CV522247
*Ta*RK	FSR	HDH	HDH*	HDH*	*A. thaliana* receptor protein kinase-like protein	CV522369
*Ta*STPK	FSR	URH	URH*	–	Rice serine/threonine protein kinase	CV522361
*Ta*G6PTP	FSR	HDH	SDH	URH	Maize glucose-6-phosphate/phosphate-translocator precursor homolog	CV522984
*Ta*ADP	RSR	DRH	DRH*	LDH*	Wheat ATP/ADP carrier protein	CV522306
*Ta*TEF	RSR	DRH	DRH	LDH*	Wheat translation elongation factor eEF-1 alpha chain	CV522596
*Ta*CDPK	RSR	DRH	DRH*	URH	Maize calcium-dependent protein kinase	CV522476
*Ta*SRP	RSR	LDH	SDH	SDH	Lactis signal recognition particle receptor protein	CV522499
*Ta*RPT3	RSR	LDH	LDH	LDH*		CV522655
*Ta*FTSH	FSL	–	–	URH	*A. thaliana* FtsH protease	CV522829
*Ta*TIF	FSL	–	HDH	URH*	Maize eukaryotic translation initiation factor 5	CV522357
*Ta*GSH	FSL	–	DRH*	URH*	Pea homoglutathione synthetase	CV522452
*Ta*EIL	FSL	–	SDH	URH	Rice ethylene-insensitive-3 like protein	CV522916
*Ta*FRLT	FSL	–	–	LDH*	A.thaliana Ferric reductase like transmembrane component family,	CV522924
*Ta*PDI3P	FSL	–	URH	LDH*	Durum wheat protein disulfide isomerase 3 precursor	CV522923
*Ta*SS	RSL	–	HDH*	LDH*	Wheat starch synthase I-1	CV523137
*Ta*TRXM	RSL	–	URH*	SDH	Wheat thioredoxin M-type	CV522532
*Ta*MTH	RSL	–	DRH	DRH*	Maize DNA cytosine methyltransferase	–

[a] FSL, RSL, FSR, RSR denotes forward subtractive library of leaves, reverse subtractive library of leaves, forward subtractive library of roots, and reverse subtractive library of roots, respectively.
[b] URH, DRH, HDH, and LDH represent the expression pattern of up-regulated in hybrid (the expression in hybrid was higher than both female and male parents), down-regulated in hybrid (the expression in hybrid was lower than its parents), high-dominant in hybrid (gene expression in hybrid was equal to the highly-expressed parent), low-dominant in hybrid (gene expression in hybrid was equal to the lowly-expressed parent). In addition, the gene expression level between hybrid and the two parents was same (SDH).
* Significant difference with P-value < 0.05.

($P < 0.05$). Another two cDNAs (*Ta*SRP and *Ta*G6PTP) did not show expression difference between hybrid and its parents on RT-PCR. Six out of 9 cDNAs (*Ta*FTSH, *Ta*TIF, *Ta*GSH, *Ta*EIL, *Ta*SS, *Ta*MTH) from the leaf subtracted libraries have same differential expression pattern in leaves as SSH, and 4 of them represented significant difference between and its parents ($P < 0.05$). Two cDNAs (*Ta*FRLT and *Ta*P-DI3P) from FSL showed LDH pattern in leaves on RT-PCR with significant difference ($P < 0.05$), opposite to the results of SSH, and another cDNA (*Ta*TRXM) expressed equally between hybrid and its parent in leaves, differing from the SSH. Overall, RT-PCR results confirmed the expression pattern of 72.2% (13 out of 18) SSH-derived cDNAs and 69.2% (9 out of 13) showed significant expression differences between hybrid and its parents. In order to test if the differential expressions are tissue or development dependent, expression profiles of the above 18 cDNAs were analyzed in roots and leaves by RT-PCR. We found that 17 out of 18 cDNAs tended to be differentially expressed in at least one tissue, 15 of them showed significant difference ($P < 0.05$). TaSRP expressed equally between hybrid and its parents both in roots and leaves. Detailed analysis revealed several expression features between wheat hybrid and its parents. Firstly, identical differential expression patterns between wheat hybrid and its parents were observed both in roots and leaves for three cDNAs (*Ta*PK, *Ta*RPT3 and *Ta*MTH), in which *Ta*PK showed high-dominant expression in hybrids (HDH) in both leaves and roots, and TaRPT3 exhibited low-dominant expression in hybrids (LDH) in both leaves and roots; Secondly, up-regulated in hybrid only in one tissue but not differentially expressed in the other (*Ta*TRXM, *Ta*EIL and *Ta*G6PTP); Thirdly, similar, but not identical expression patterns were found in roots and leaves for four cDNAs (*Ta*ADP, *Ta*TEF, *Ta*PRBP and *Ta*TIF). For example, *Ta*TEF showed DRH in roots and LDH in leaves; Fourthly, opposite expression patterns were displayed for four cDNAs (*Ta*CDPK, *Ta*PDI3P, *Ta*SS and *Ta*GSH) in roots and leaves. For example, TaCDPK was up-regulated in hybrid leaves, but down-regulated in hybrid roots; finally, tissue-specific expressions were demonstrated for 3 cDNAs (*Ta*FTSH, *Ta*FRLT and *Ta*STPK), in which case *Ta*FTSH and *Ta*FRLT showed higher-level expression in leaves than that of roots.

Characterization complete ORF and spatio-temporal expression analysis

To test whether differentially expressed cDNA fragments truly represent the putative genes and provide a basis for further analysis of their functions, *in silico* cloning approach was applied to obtain full-length cDNA sequences from novel gene sequences and the sequences were confirmed by RT-PCR. We obtained 10 cDNAs containing complete open reading frames (ORF) according to bioinformatic analysis, temporarily termed as *Ta*20B4, *Ta*20B5, *Ta*20B7, *Ta*UBC, *Ta*IPP, *Ta*LRRP, *Ta*ARF, *Ta*HD, *Ta*RPS13, and *Ta*14-3-3 (Accession number: AY736118 to AY736127) (Table 6). Except for *Ta*ARF and *Ta*14-3-3, the remaining 8 ORFs represented novel sequences identified in wheat.

Since our SSH libraries were constructed from leaf and root tissues of seedling stage, we want to

Table 6. Ten cDNAs with complete open reading frame.

Gene name	Length (bp)	ORF (bp)	Peptides (aa)	Homology	Accession number
*Ta*20B4	654	1–642	213	Rice 20S proteasome beta 4 subunit	AY736118
*Ta*20B5	860	10–849	279	Rice 20S proteasome beta 5 subunit	AY736119
*Ta*20B7	694	42–689	215	Rice 20S proteasome beta 7 subunit	AY736120
*Ta*UBC	460	11–457	148	Rice ubiquitin-conjugating enzyme	AY736121
*Ta*IPP	909	41–901	286	Rice inositol phosphatase-like protein	AY736122
*Ta*LRRP	740	54–710	218	Rice leucine-rich repeat protein	AY736123
*Ta*ARF	583	28–573	181	Rice ADP-ribosylation factor	AY736124
*Ta*HD	1417	36–1412	458	Maize histone deacetylase	AY736125
*Ta*RPS13	494	35–490	151	Maize cytoplasmatic ribosomal protein S13	AY736126
*Ta*14-3-3	804	4–795	263	Wheat 14-3-3 protein	AY736127

know if the differential expression patterns between hybrid and its parent can also be detected in other tissue and developmental stages. Ten genes were used to determine whether the differential expression pattern between hybrid and its parent are depended on tissues and developmental stages. Expression patterns between hybrid and its parents of these 10 genes in different tissues and developmental stages (tillering stage, jointing stage and booting stage) (Figure 5) were examined by RT-PCR. For eight of these genes, variations in differential expression patterns between hybrid and its parents were observed in different developmental stages and tissues tested. We found two genes exhibited most dramatically developmental variation in their expression patterns. *Ta*20B5 showed URH pattern in shoots and leaves at jointing stage but LDH pattern in roots at tillering stage and DRH pattern in spikes of booting stage. Ta20B7 showed HDH pattern in most of the tissues but DRH pattern in shoots at jointing stage. It is worthy to note that the transcription of two genes (*Ta*20B4 and *Ta*HD) is more abundant in hybrid F_1 in all tissues examined as compared to its parents, suggesting that these genes are over-expressed or high-dominantly expressed in hybrid F_1.

Growth-stage-specific expression patterns can provide new insights and hypotheses concerning the possible role of differentially expressed genes. In this paper, the spatio-temporal expression analysis of 10 genes with complete ORF was also analyzed by RT-PCR. The spatial expression

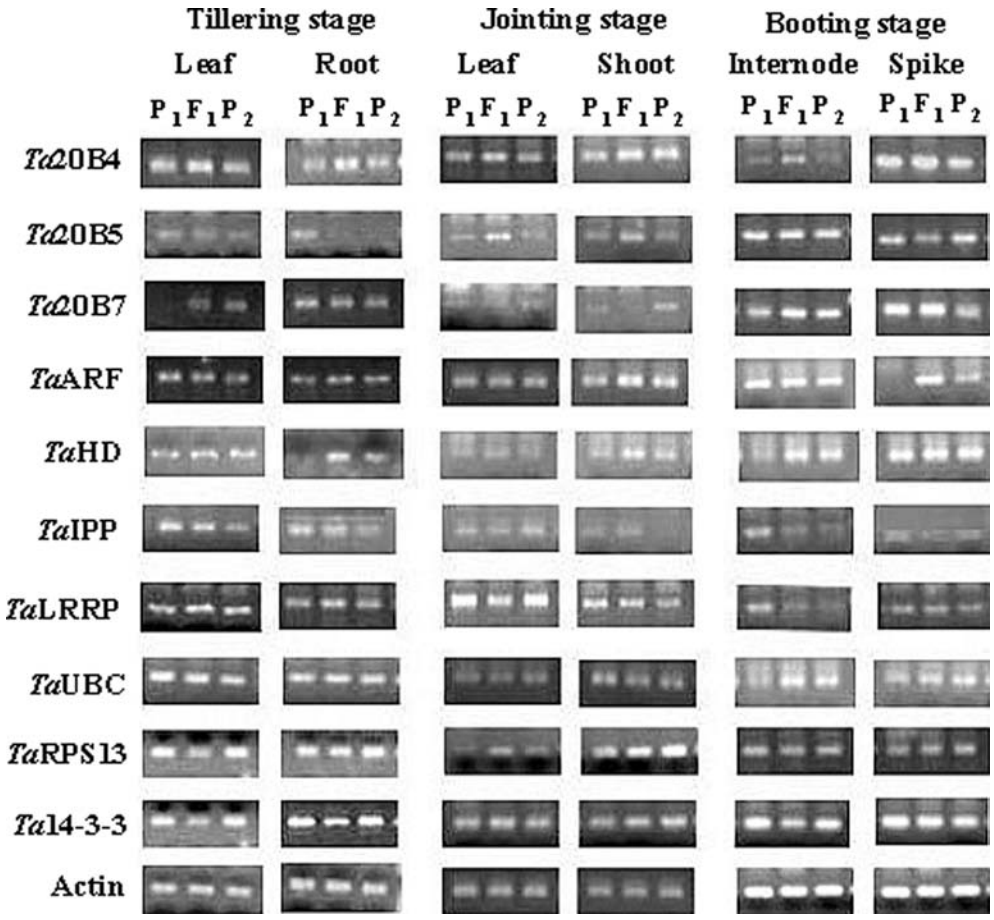

Figure 5. Expression of 10 genes with putative open reading frame between hybrid and its parents in different tissues (leaf, root, shoot, internode and spike) and different developmental stages (tillering, jointing and booting stage) by semiquantitative RT-PCR. A wheat actin gene was used to normalize the amount of template added in PCR reaction. P_1 represented female parent 3338; F_1 represented hybrid 3338/2463, P_2 represented male parent 2463. Five microliters of PCR reactions were separated in a 1.0% agarose gel.

patterns of the 10 ORFs from roots, shoots and leaves at the trefoil stage of common wheat 3338 could be classified into five groups (A, B, C, D and E). Groups A (*Ta*IPP, *Ta*20B5and *Ta*14-3-3), B (*Ta*HD and *Ta*RPS13) and C (*Ta*LRRP and *Ta*20B7) represented genes which are preferentially expressed in leaves, shoots and roots, respectively, while the genes of group D (*Ta*20B4) were highly expressed both in leaves and roots and the group E genes (*Ta*UBC and *Ta*ARF) were expressed throughout roots, shoots and leaves. RT-PCR expression profiles in spikes at the floral ontogeny (including booting, heading and anthesis stage) demonstrated that five genes (*Ta*20B4, *Ta*20B5, *Ta*20B7, *Ta*RPS13 and *Ta*HD) were highly expressed at the booting, and then gradually decreased at the heading and anthesis phases. The transcriptions of *Ta*IPP were more abundant at the anthesis stage and the other four genes (*Ta*UBC, *Ta*ARF, *Ta*14-3-3 and *Ta*LRRP) showed a constitutive expression patterns throughout the floral ontogeny stages (Figure 6).

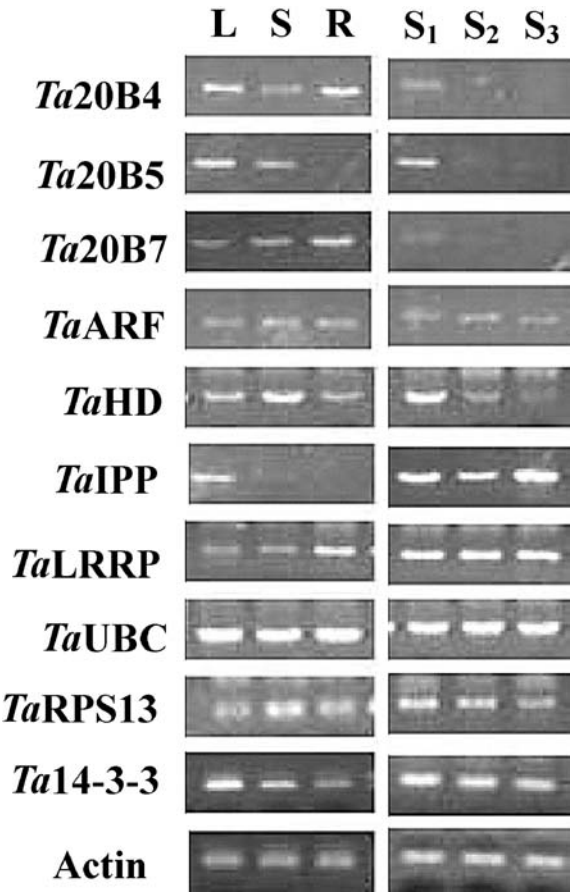

Figure 6. Tissue-specific expression analyses of 10 genes with putative open reading frame. A wheat actin gene was used to normalize the amount of templates added in PCR reactions. L, leaves of seedling in trefoil stage; S, shoot of seedling in trefoil stage; R, roots of seedling in trefoil stage. S1, spike in booting stage, S2, spike in heading stage, S3, spike in anthesis stage. Five microliters of PCR reactions were separated in a 1.0% agarose gel.

Discussion

Suppression subtractive hybridization combined with in silico cloning provides an efficient method for identification of differentially expressed genes between hybrid and its parent

In order to provide further insight into understanding the molecular mechanism of heterosis, several groups and our laboratory have been working on detecting differential gene expression and isolating differentially expressed genes between hybrids and their parents (Xiong *et al*., 1998; Wu *et al*., 2003; Sun *et al*., 2004). However, the complex phenomena of heterosis could not be characterized without systematic examination of differentially expressed genes between hybrid and its parent. Innovative tools, such as suppression subtractive hybridization (SSH) and microarray, allow such a more global analysis of differential gene expression. In this paper, we modified the PCR-based subtractive method by mixing equal amount of poly(A) RNA of two parents for SSH, and employed 3.5% nondenaturing polyacrylamide gels to detect the fragments of same length for eliminating possible redundant cDNA clones. Two modifications have been made for the PCR-based SSH in the study. First, equal amount of parental poly(A) RNAs were mixed as driver/tester for subtraction hybridization to hybrid cDNAs (tester/driver). This mixture could result in a higher estimation of up-regulated in hybrid (URH) and low estimation of high dominant in hybrid (HDH). However, reverse-Northern analysis showed 68.2% of the SSH-derived roots libraries cDNAs displayed differential expression patterns between hybrid and its parents. Further semi-quantitative RT-PCR analysis on 18 SSH-derived cDNAs from the root and leaf libraries revealed 72.2% of the cDNAs represent real differential expressions between hybrid and its parents. Second, a 3.5% nondenaturing polyacryl-

amide gels have been employed to eliminate possible redundant cDNA clones of similar insert size, resulting 926 cDNAs were selected for sequencing from 2500 colonies, 748 nonredundant cDNAs were obtained with low redundancy. With these two modifications, SSH provide a fast and reliable procedure to detect differential gene expressions between wheat hybrid and its parental inbreds. However, all of the cDNAs identified by SSH were expressed both in hybrid and its parents, mostly exhibited predominant expression in hybrid or its parents, suggesting that the gene expression difference between hybrid and its parents mostly happened on quantitative not on qualitative level.

In silico cloning provides a cost-effective way to obtain complete ORFs based on the public sequence data. We obtained 10 complete ORFs by this approach after performing PCR amplification, cloning and sequencing the PCR products based on bioinformatic analysis of overlapping EST or protein sequences in the GenBank. In this study, the combination of SSH and *in silico* cloning provides an extremely efficient method for identification a batch of differential expressed genes with putative ORF between hybrid and its parents, which enable us to conduct further analysis of their functions and possible roles in heterosis.

Differential expression patterns and heterosis

Previous studies using multiple hybrids and their corresponding parents indicated that some differential expression patterns were significantly correlated with heterosis observed in one or more agronomic traits (Xiong *et al*., 1998; Sun *et al*., 2004). In wheat, Sun *et al*. (2004) demonstrated that among the four differential expression patterns, BPnF1 (similar to LDH in this study), F1nBP (similar to URH) and UPF1 (similar to HDH) were significantly correlated with heterosis observed in six, seven and three agronomic traits, respectively. In this study, among the 316 differentially expressed cDNAs validated by reverse-Northern blot, the numbers of cDNAs representing the four differential expression patterns, i.e., URH, DRH, HDH and LDH, which were equivalent to overdominance, underdominance, complete dominance and recessive in quantitative genetics theory, were 71, 89, 81 and 75, respectively. During last decade, with the advent of QTL mapping, three types of QTL interactions were proposed to contribute to heterosis in different studies, i.e., overdominance in maize (Stuber *et al*., 1992), dominance in rice (Xiao *et al*., 1995) and epistasis in rice (Yu *et al*., 1997). More recently, however, Hua *et al*. (2003) proposed that all kinds of genetic effects, including partial-, complete- and overdominance, as well as epistasis contributed to heterosis in a rice 'immortalized F2' population, indicating that these genetic effects were not mutually exclusive in the genetic basis of heterosis. In this study, at gene expression level, nearly equal number of cDNAs for each of the four differential expression patterns were represented in the SSH library. Given that at least three differential expression patterns, URH, HDH and LDH were correlated with heterosis in our previous study, the combination of overdominance, complete dominance and recessive effects could contribute to heterosis, which might provide evidence at expression level for the finding of Hua *et al*. (2003). However, it should be noted that we still do not know whether all the differentially expressed genes are contributors to the heterosis or some of them are merely the results of hybridization. Further studies are needed to determine the functional relationship between differentially expressed genes and heterosis in specific traits.

Genes of diverse categories are differentially expressed between hybrid and its parents

It is suggested that gene expression changes between hybrid and its parents may be responsible for heterosis (Xiong *et al*., 1998; Sun *et al*., 2004). Previous studies (McDabiel, 1985; Romagnoli *et al*., 1990) have demonstrated that several biochemical properties of hybrid exceed those of parental lines, such as mitochondrial oxidation and phosphorylation, nucleic acid synthesis, nitrogen metabolism, plant hormone levels, and relative enzymes activities. The differentially expressed genes identified in this study involved diverse physiological process, including metabolism, photosynthesis, transport, materials transportation, and signal transduction, providing direct evidences of hybrid vigor on transcriptional level. Further functional characterization of these differentially expressed genes might shed lights on understanding of the molecular basis of wheat heterosis.

It has been suggested that differential gene expression and protein synthesis may be the basis

of heterosis in plants (Romagnoli *et al*., 1990). We found several ribosomal protein genes are differentially expressed between hybrid and its parents. In eukaryotes, four ribosomal RNAs and about 80 ribosomal proteins contribute to the assembly of the ribosome, which is the machinery for protein biosynthesis. Strong stimulation of the expression of genes encoding components of the transnational apparatus is observed in cycling cells (Gao *et al*., 1994) and mutations in several genes encoding ribosomal proteins in *Arabidopsis* result in morphological alterations and a significant delay in development (Ito *et al*., 2000; Weijers *et al*., 2001), indicating the important roles of ribosomal proteins to plant development.

The regulatory mechanism of differential transcriptions between hybrid and it parents remains unknown. Several regulatory processes at the transcription and post-transcription level, as well as epigenetic control, such as DNA methylation, histone acetylation, chromatin remodeling may be related to the transcription changes. It has been suggested that altered regulatory interactions, e.g. through differences in the expression of specific transcription factors, may be the basis of heterosis in plants (Osborn *et al*., 2003). Recent research also suggested the differential expression of transcription factors might contribute to heterosis (Wu *et al*., 2003). In this study, the differential expression of nine transcription factors was observed between hybrid and its parent.

RNA–protein interactions play important roles in post-transcriptional expression regulation through pre-mRNA capping, polyadenylation, splicing, exporting mRNA from nucleus and maintaining mRNA stability. The expressions of RNA-binding proteins tend to be tissue specific, developmentally regulated, and responsive to a wide range of external stimuli. A hybrid-specific expressed RNA-binding protein gene in wheat seedlings has been reported (Ni *et al*., 2000). A putative pumilio/Mpt5 family RNA-binding protein (*Ta*PRBP) was found expressed equally to the highly expressed parent in hybrid (Table 5). The putative amino acid sequence of this gene was homologous to the C-terminal of barley pumilio protein (AAM22812). Pumilio proteins are sequence specific RNA-binding proteins capable of recognizing specific nucleotide sequence in the 3′UTR of target mRNAs and acting as translational repressors during development and differentiation (Edwards *et al*., 2000).

Histones are essential components of eukaryotic chromosomes and play crucial roles in the maintenance of chromosomal integrity (Kornberg and Lorch, 1999). It was first reported three decades ago that synthesis of histone proteins occurs primarily during S-phase of the cell cycle (Robbins and Borun, 1967). Subsequently, a large body of research has established that the S-phase-specific increase in histone protein levels results directly from the accumulation of histone mRNA in S-phase, which in turn is controlled at the level of transcription, pre-mRNA processing, and mRNA stability (Zhao *et al*., 2000). A few genes encoding wheat histone H1, H2B-1, H2B-2, H2B-6 and H2B153 were found up-regulated in hybrid, which might be responsible for the active metabolism in hybrid. A higher H1 and H2B mRNA accumulation has been reported in organs which contain meristematic tissue and/or which have a high proportion of actively cycling cells in gibberellin-deficient tomato (Van den Heuvel *et al*., 1999). The higher-level expression of histones in hybrid may contribute to the high rate of hybrid growth. Moreover, histones participate in transcription regulation by manipulating DNA condensation, thereby influencing the access of transcription factors to promoters. Histone acetylation/deacetylation catalyzed by histone acetyltransferase/deacetylase affect the accessibility of RNA polymerase to chromatin DNA. The rice *Os*HDAC1, histone deacetylase gene, involved genome-wide programming of gene expression and its overexpression in transgenic rice leads to a range of novel phenotypes, such as increased growth rate and altered plant architecture (Incheol *et al*., 2003). The *Ta*HD, wheat homology of *Os*HDAC1 was up-regulated in hybrid. Further characterization of the possible link between higher expression of *Ta*HD in hybrid and hybrid vigor will be untaken in our lab.

Roots system can be used as a model trait for the investigation of the molecular basis of heterosis

The main functions of higher plant root system are to anchor the plant, to explorer the soil for water and mineral nutrients, and to produce

several growth controlling substances. As compared to the well-studied aerial part like plant height and grain yield, the heterosis of plant root system was poorly understood. Meyer *et al.* (2004) reported that root hair length of the hybrid F_1 in Arabidopsis surpassed that of both parents. We found that 5 of 10 root traits shown significant heterosis (MHP > 20%) and the MPH of wheat roots biomass (35.3% in nutrient solution, 75.3% in vermiculite) is much higher than that of aerial parts (23.3% in nutrient solution, 39.9% in vermiculite). A better-developed hybrid root system could potentially lead to increased nutritional uptake to support elevated growth rates, thus contributing to heterosis.

Differential gene expressions between hybrid and its parental inbreds in roots may play an important role in root heterosis. By analyzing mRNAs and proteins produced in primary root tips, Romagnoli *et al.* (1990) found that patterns of gene expression and protein quantity were altered in a heterotic maize hybrid and its parental lines. Ni *et al.* (2002) found that more differentially expressed genes were observed in the primary roots than in the seedling leaves. In this study, we performed screening of differentially expressed genes between wheat hybrid and its parents in roots of tillering stage by SSH technique. Sequence analysis and database search revealed that differentially expressed genes involved in metabolism, signal transduction, and resistance. It is worthy to note that a subset of 11 cDNAs belong to the carrier or transporter, such as zinc transporter, amino acid transporter, was uniquely identified from root subtractive libraries, indicating that the ability of hybrid for nutrients transport is different from its parents, which may contribute to the root biomass heterosis.

Roots are relatively simple organs and root morphogenesis normally occurs in a reiterative and uniform fashion without any significant developmental transition, which can be a useful system for understanding organ development (Aeschbacher *et al.*, 1994). The significant heterosis of hybrid root and the differentially expressed genes as detected in this study and elsewhere (McDaniel, 1985; Ni *et al.*, 2002) may open a new window in understanding the molecular basis of heterosis.

Similar changes in gene expression patterns are observed both in hybrid F_1 and polyploid

Polyploidy has played an important role in the evolution of higher plants and 50–70% of all angiosperm species are of polyploid origin (Masterson, 1994). During the last two decades, molecular data have provided new insights into understanding the mechanism and evolutionary aspects of polyploidy (Wendel, 2000; Osborn *et al.*, 2003). Allopolyploids represent a special type of hybrid. The two parental genomes in an allopolyploid, called homologous, undergo little intergenomic recombination and thus maintain their integrity through sexual generations (Comai, 2000a). Allopolyploidy is often advantageous, and one factor that favors allopolyploids may be heterosis generated by the combination of homologous genes (Allard *et al.*, 1993; Jiang *et al.*, 1998). Polyploidy alone can affect the expression of certain genes. Previous studies reported that in yeast expression of several genes is affected by ploidy level, and the suppression of certain G1 cyclins is thought to be responsible for the increase in cell size that accompanies polyploidy (Galitski *et al.*, 1999). In maize, the ploidy level can either increase or decrease expression, depending on the particular gene (Guo *et al.*, 1996). In Arabidopsis, a case of transgene silencing is correlated with tetraploidy (Mittelsten *et al.*, 1996). To establish themselves as successful species, the genomic structure and gene expression of newly formed allopolyploids often change through rearrangements in noncoding genomic DNA, through epigenetic changes and regulation of gene expression (Kashkush *et al.*, 2002; Kashkush *et al.*, 2003). Kashkush *et al.* (2002) found that different genes (rRNA genes and genes involved in metabolism, disease resistance, and cell cycle regulation and retroelements) showed alteration in expression between a synthetic wheat allotetraploid F_1 and its two diploid progenitors. In this study, we also found the similar types of genes (such as genes involved in metabolism, disease resistance) altered in expression between hybrids and their parents. Furthermore, allopolyploid formation may be associated with epigenetic changes. Epigenetic interactions in the hybrids may occur between homologous genes or between genes and related sequences within heterochromatin (Comai *et al.*,

2000b). We also observed the differential expression in genes involved in epigenetic regulation (*Ta*MTH and *Ta*HD). The phenomenon of heterosis observed in hybrid F_1 could also be considered as a type of phenotype evolution, which occurs in hybrids produced through hybridization of two parents. Similar changes in gene expression pattern were observed in both types of hybrids, indicating that similar mechanisms for gene expression and regulation may be responsible for both polyploidy evolution and heterosis. In fact, the results reported here strongly support this hypothesis, and we believe that further comparison study will be very helpful for understanding of molecular basis underlying heterosis. Furthermore, insight into genome function gained from the study of allopolyploidy may be applicable to hybrids of any type and may even elucidate positive interactions, such as those responsible for hybrid vigor.

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