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ORIGINAL PAPER

Heterosis in root development and differential gene expression between hybrids and their parental inbreds in wheat (*Triticum aestivum* L.)

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Abstract In spite of commercial use of heterosis in agriculture, the molecular basis of heterosis is poorly understood. In this study, heterosis was estimated for eight root traits in 20 wheat hybrids derived from a NC Design II mating scheme. Positive mid-parent heterosis was detected in 96 of 160 hybrid–trait combinations, and positive high-parent heterosis was detected in 79 of 160 hybrid-trait combinations. Improved differential display was used to analyze alterations in gene expression between hybrids and their parents in roots at the jointing stage. More than 990 fragments were repeatedly displayed, among which 27.52% were differentially expressed between hybrids and their parents. Four differential expression patterns were observed. Thirty differentially expressed cDNA fragments and three genes with open reading frames were cloned, and their expression patterns were confirmed by reverse-northern blot and semi-quantitative RT-PCR analysis, respectively. We concluded that these differentially expressed genes, though mostly with unknown function, could play important roles for hybrids to demonstrate heterosis in root system traits.

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Introduction

Heterosis, or hybrid vigor, refers to the phenomenon that hybrids exhibit greater biomass, speed of development and fertility than the better of the two parents. Hybrid cultivars have been used commercially in many crop plants, and made significant contributions to the world food supply (Duvick 1997). The genetic basis of heterosis has been discussed for nearly a century, and the classical genetic explanations for heterosis centered on two hypotheses, namely, the dominance hypothesis (Davenport 1908; Bruce 1910) and the over-dominance hypothesis (East 1908; Shull 1908). QTL mapping studies have provided further support to dominance (Xiao et al. 1995), over-dominance (Stuber et al. 1992) and epistasis (Yu et al. 1997; Li et al. 2001; Luo et al. 2001) models. Hua et al. (2003) suggested that, at the population level, all kinds of genetic effects could contribute to the genetic basis of heterosis.

In spite of these genetic studies, the molecular mechanism of heterosis remains to be revealed.

Although the genome of an F_1 hybrid is derived from its parents, hybrid performance is quite different from its parents. Therefore, it is reasonable to speculate that changes in gene expression may occur in hybrids as compared to its parents, and such changes, if any, should be responsible for the heterosis observed in the F_1 hybrid (Birchler et al. 2003; Sun et al. 2004). Earlier reports demonstrated that some proteins and mRNAs are differentially synthesized and expressed in root tips between a maize hybrid and its parents (Romagnoli et al. 1990), and the mean quantities of mRNA for 35 genes were higher in a highly heterotic hybrid than in a non-heterotic hybrid (Tsaftaris 1995; Tsaftaris and Kafka 1998). Differences in mRNA quantity and patterns between heterotic hybrids and their parents were also detected in maize and rice (Chen et al. 1996, 1997). Studies also indicated that the differential gene expression patterns in leaves were correlated with heterosis for agronomic traits in rice (Xiong et al. 1998) and wheat (Wu et al. 2003; Sun et al. 2004). Non-additive gene expression has also been observed in maize hybrids and gene regulatory interactions were suggested to contribute to this non-additivity (Auger et al. 2005).

Roots acquire water and nutrients from the environment (Schiefelbein et al. 1997). The root system has recently been the focus of research interest as a useful system for understanding organ development since root is a relatively simple organ and its morphogenesis normally occurs in a reiterative and uniform fashion without any significant developmental transition (Aeschbacher et al. 1994). Studies have shown that root length, root surface area (RSA), root biomass, root volume (RV), and root/shoot ratio (RSR), as important factors of uptake efficiency, vary among cultivars (Fohse et al. 1988; Jackson et al. 1997; Samad et al. 2002). Better performance stability was also observed in wheat hybrids compared to pure line varieties, and root development was proposed to contribute to this stability (Brouwer 1983). However, there has been no systematic investigation on heterosis in root traits of wheat.

This study was undertaken to estimate the mid-parent heterosis (MPH) and high-parent heterosis (HPH) for 20 wheat hybrids derived from NC Design II mating scheme and their corresponding parents in eight root traits, i.e., total root length (TRL), RSA, root average diameter (RAD), root tips no. (RTN), longest root length (LRL), RV, root dry weight (RDW) and RSR. We also conducted a differential display reverse transcription (DDRT) analysis to investigate the relationship between differential gene expression patterns and heterosis in root system traits.

Materials and methods

Genetic materials

Four elite wheat lines (3338, 3235, 227 and 101) were used as female parents and crossed with each of five male parents (390, 6554, 411, 3214 and 8790) in a NC Design II mating scheme, resulting in 20 hybrids. These nine genotypes were previously used in our studies on hybrid performance (Wu et al. 2001) and selected in this study.

Growing conditions

Pre-germinated seeds were cultivated on autoclaved sand and irrigated with a nutrient solution (Broughton and Dilworth 1971) containing (μM): $CaCl_2$ (1,000), KH_2PO_4 (500), $MgSO_4$ (250), K_2SO_4 (250), H_3BO_3 (2), $MnSO_4$ (1), $ZnSO_4$ (0.5), $CuSO_4$ (0.2), $CoSO_4$ (0.1), Na_2MoO_4 (0.1) and supplemented with 500 μM KNO_3. Iron was supplied as 20 μM NaFe (III) EDTA, and the pH was adjusted with NaOH (1 M) to 6.5. The seedlings were removed from the sand and transferred to nutrient solution in capped porcelain pots at the three-leaf stage (two seedlings per pot). Three replicates were used in this study. Each pot contained 1,000 ml of nutrient solution and was aerated. Experiments were carried out in a growth chamber at a relative humidity of 75% and 26/20°C day and night temperature, 14-h day length. Mixed cool-white fluorescent tubes and incandescent bulbs provided light in the growth chamber. At the jointing stage, roots were sampled from the seedlings grown in the growth chamber conditions to measure root traits and estimate heterosis. Another four seedlings of each genotype were transplanted to PVC tubes and the tubes were placed in the field, and the diameter and length of each tube were 16 and 100 cm, respectively. Roots harvested from PVC tubes were used for RNA exaction. Since field root harvesting is challenging, special care was taken to ensure that roots were dug up carefully and that root systems were as intact as possible. Roots were collected at jointing stage and stored at −80°C for RNA extraction.

Root system quantification

Harvested roots were transferred to ice to prevent dehydration and taken to the laboratory where they were processed for analysis. 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 for scanning to a 30 cm×20 cm plexi-glass plate where they floated in clear water and were carefully dispersed into individual lateral roots and secondary roots with forceps as far as possible to prevent overlapping (Harris and Campbell 1989). Care was taken to exclude the sides of the tray from the window area to avoid erroneous counts. Each root was scanned twice, in two directions to assure scanning of all roots and the two measurements were averaged. Although root systems develop a three-dimensional form in the soil, roots were measured in two dimensions in this study. The following root morphology parameters were measured: TRL (cm), RSA (cm^2), RAD (mm), RTN and RV (cm^3). Dry weight (dried at 60°C for 72 h for dry weight determination) of shoot and root of the plants were taken and used for calculation of RDW (g) and RSR. LRL (cm) was measured manually.

RNA extraction

Five grams of root samples for each of above 29 genotypes were ground to the fine powder under liquid nitrogen, and transferred into a 50-ml Eppendorf tube. Ten milliliters of pre-warmed (90 °C) extraction buffer [containing 100 mM LiCl, 1% SDS, 10 mM EDTA-Na_2 and 100 mM Tris–HCl (pH 9.0)] were added and mixed with an equal volume of phenol (pH 8.0). After adding 4 ml of chloroform, tube was shaken for 10 min and centrifuged at 12,000 rpm for 20 min at 4°C. The supernatant was carefully transferred to a new micro-centrifuge tube and extracted twice by equal volume of chloroform. The supernatant was collected and precipitated with one-third volume of 8 M LiCl for 16–18 h at 4°C. The tube was centrifuged at 12,000 rpm for 20 min at 4°C, and the supernatant was discarded. The precipitate was washed in 2 M LiCl and 80% ethanol, and eluted in 40 µl RNase-free water. Total RNA was digested to eliminate residual DNA with DNase I (Promega, Madison, WI, USA) for 30 min and extracted twice by chloroform.

Reverse transcription

Two micrograms of RNA of each sample were used for 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 MMLV reverse transcriptase (Promega, Madison, WI, USA) and 50 pmol one-base-anchor-oligonucleotide $HT_{11}A$, $HT_{11}C$ or $HT_{11}G$ (H represents AAGC). Reverse transcription was performed for 60 min at 37°C and then a final denaturation step was conducted at 95°C for 5 min.

PCR amplification of cDNA

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

3′ end-anchored primers
$HT_{11}A$: 5′-AAGCTTTTTTTTTTTA-3′
$HT_{11}C$: 5′-AAGCTTTTTTTTTTTC-3′
$HT_{11}G$: 5′-AAGCTTTTTTTTTTTG-3′
5′ end oligo-nucleotide primers
DD10: 5′-TGCCGAAGCTTTGGTAGC-3′
DD18: 5′-TGCCGAAGCTTTGGTCAC-3′
DD19: 5′-TGCCGAAGCTTTGGTCAG-3′
DD20: 5′-TGCCGAAGCTTTGGTCAT-3′
DD32: 5′-TGCCGAAGCTTGGAGCTT-3′

In order to test reproducibility, we conducted two independent PCR reactions for each sample. Two microliters of each cDNA were subjected to PCR reaction employing the corresponding one base anchor oligo-nucleotide along with one of the DD (differential display) random primers, 1.5 mM $MgCl_2$, 0.20 mM dNTP and 1 U Taq polymerase in a 20-µl final volume. To verify that there was no DNA contamination in the RNA samples, a negative control was prepared without reverse transcription. The PCR program was performed as follows: one cycle at 94°C for 1 min; 40°C for 4 min; 72°C for 1 min, followed by 40 cycles: 94°C for 45 s; 60°C for 2 min; 72°C for 1 min. One final step was at 72°C for 8 min.

Electrophoresis

PCR products were separated on 0.4-mm thick, 4% denaturing poly-acrylamide sequencing gels in a temperature-regulated Bio-Rad Sequencing System (Bio-Rad, Santa Rosa, CA, USA) at 50°C. Gels were silver-stained to visualize fragments that were differentially expressed in root between wheat hybrids and their parents at the jointing stage. Only cDNA fragments that were differentially displayed in both of the two independent PCR reactions were used for further analysis. The percentages of the different expression patterns were estimated for each of the 20 hybrids.

Statistical analysis

Mid-parent heterosis and HPH were calculated as: MPH = (F_1 − mean P)/mean P in percent, HPH = (F_1 − high P)/high P in percent (Falconer and Mackay 1996). Analysis of variance (ANOVA) was performed for each trait in both hybrids and parental inbred lines using SAS v. 8.2 (SAS Institute 1999).

Cloning, sequencing and reverse-northern blot

Bands that were different between one heterotic hybrid (3,338×6,554) and its parental inbred lines were excised from gels and re-amplified using the following PCR conditions: 3 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 8 min. The differentially expressed cDNA fragments were cloned into pGEM-TEasy vector (Promega, Madison, WI, USA), the sequenced and blasted in NCBI (http://www.ncbi.nlm.nih.gov) using blastx and blastn at the GenBank nr database firstly. If no hits in the database, we blasted the sequences against the EST database to gain more information. Reverse-northern analysis was performed using dot blot. Total RNA of this hybrid and its parental inbreds were ^{32}PdCTP-labeled and hybridized to Hybond N^{+} membrane (Amersham, UK) according to manufacturer's recommendations.

Cloning and RT-PCR analysis of cDNA sequence with complete open reading frame

In silico cloning started from cDNA sequences obtained in the current work, and overlapping dbEST sequences from GenBank were assembled into contigs to obtain open reading frames (ORFs). Gene-specific primers were designed based on the N- and C-terminal sequences of ORFs and used for obtaining the corresponding ORFs in roots: Primers ANN-F (5′-ATGGCG AGCCTGAGCGTGCC-3′) and ANN-R (5′-TTAGCG GTCGCGGCCGAC GA-3′) were used for *TaANN1*; Primers prolamine box binding factor (PBF)-F (5′-CT ATACTCCATACTACCCTTCGTT CACC-3′) and PBF-R (5′-AATGACCTCCATTTCCCATTTTCTT-3′) were used for *TaPBF2*; Primers COB-F (5′-CCGCAC ATGCTTTAAGTCCCACTC-3′) and COB-R (5′-GG TCTCGTTTCATCATACGTAATA-3′) were used for *TaCOB1* gene. Semi-quantitative RT-PCR using gene-specific primers are carried to confirm the expression patterns of cloned genes between the wheat hybrid and its parents. The following primers for tubulin were used as control: TUB-F (5′-ACCGCCAGCTCTTCC ACCCT-3′) and TUB-R (5′-TCACTGGGGCATAG GAGGAA-3′).

Results

Heterosis in root system traits

Eight root characters, i.e., TRL, RSA, RAD, RTN, LRL, RV, RDW and RSR, were measured for each of the 20 hybrids and their corresponding parents. ANOVA indicated that differences among 20 hybrids as well as among nine parents were highly significant (P<0.01) for all eight root traits, whereas differences between hybrids and parents were highly significant for seven of the eight root traits (Table 1). Significant positive MPH was found in 96 out of 160 hybrid–trait combinations (60%), while significant positive HPH was found in 79 out of 160 hybrid–trait combinations (49%) (Table 2). Across the 20 hybrids, mean MPH and HPH values were 46.92 and 30.85% for TRL, 54.12 and 39.33% for RSA, 71.46 and 55.53% for RAD, 33.39 and 8.57% for RTN, 19.60 and 10.73% for LRL, 46.84 and 26.87% for RV, 36.73 and 16.12% for RDW and 6.66 and −11.03% for RSR, respectively. The frequencies and levels of heterosis observed were higher than those we had observed in a previous study on above-ground traits (Wu et al. 2001).

Mid-parent heterosis and HPH values differed greatly from one character to another for a given hybrid, and also varied widely among the 20 hybrids for a given trait (Table 2). On average across the 20 hybrids, RAD showed the highest mean MPH (71.46%) and HPH (55.53%), followed by RSA (54.12, 39.33%) and TRL (46.92, 30.85%), while RSR showed the lowest MPH (6.66%) and HPH (−11.03%). It was found that four hybrids 3338/390, 3338/6554, 3235/6554 and 227/8790 exhibited positive MPH in all eight root traits, while one hybrid, 3338/6554, exhibited positive

Table 1 Analysis of variance of eight root traits

Sources	*df*	MS							
		Total root length	Root surface area	Root average diameter	Root tips no.	Longest root length	Root volume	Root dry weight	Root/shoot ratio
Crosses	19	8,143,411*	160,382*	13.16*	64,339,073*	491.6*	7.51*	0.0219*	0.00655*
Parents	8	2,403,385*	39,676.7*	2.445*	46,433,171*	237.3*	5.66*	0.0153*	0.01465*
Parents vs. crosses	1	57,534,551*	1,763,467*	146.8*	1.41E+08*	1,693.3*	70.52*	0.0976*	7.70e−06
Error	58	23,521.77	4,825.57	0.038	267,085.8	2.8	0.08	0.0007	0.00045

*Significant at $P < 0.001$

Table 2 Mid-parent heterosis and high-parent heterosis (values in parenthesis) in eight root traits at jointing stage

Crosses	Total root length (%)	Root surface area (%)	Root average diameter (%)	Root tips no. (%)	Longest root length (%)	Root volume (%)	Root dry weight (%)	Root/shoot ratio (%)
3338/390	35.83***	66.22***	80.25***	19.26***	70.45***	131.88***	105.56***	26.19*
	(33.61***)	(56.06***)	(48.28***)	(−0.96)	(66.67***)	(105.13***)	(94.74***)	(−4.92)
3338/6554	74.17***	84.35***	150.7***	37.82***	58.24***	37.08***	44.90***	88.12***
	(72.61***)	(82.19***)	(143.58***)	(23.83***)	(56.52***)	(22.00***)	(18.33*)	(64.37***)
3338/411	8.59*	8.59	3.04	−19.31***	20.00***	81.82***	108.89***	−4.24
	(−8.85**)	(−6.94)	(−10.7*)	(−36.31***)	(5.00*)	(50.00***)	(80.77***)	(−36.73***)
3338/3214	−25.74***	−28.26**	−13.48***	−38.53***	28.00***	82.02***	44.68***	21.63*
	(−27.87***)	(−28.34**)	(−18.80***)	(−48.62***)	(16.36***)	(62.00***)	(21.43**)	(−14.61*)
3338/8790	56.7***	58.4***	69.33***	−7.6**	5.45*	−11.33***	20.83*	2.82
	(44.53***)	(48.46***)	(63.93***)	(−10.68***)	(−10.77***)	(−28.00***)	(0.00)	(−28.62***)
3235/390	27.62***	35.82***	18.21***	−23.96***	−14.89***	102.5***	20.48	2.44
	(26.41***)	(26.51**)	(3.90)	(−29.96***)	(−21.57***)	(62.00***)	(2.04)	(−3.89)
3235/6554	67.96***	77.08***	163.71***	49.94***	38.14***	100***	17.43*	9.25
	(65.13***)	(73.54***)	(137.74***)	(48.79***)	(31.37***)	(100.00***)	(6.67)	(−3.11)
3235/411	75.23***	72.29***	64.3***	76.33***	4.5	25.45***	−16.83	−32.59***
	(50.28***)	(46.62***)	(33.43***)	(53.19***)	(−3.33)	(15.00***)	(−19.23*)	(−47.57***)
3235/3214	2.35	2.66	37.58***	18.2***	45.28***	22***	−0.95	−17.82*
	(2.02)	(1.90)	(35.57***)	(9.69**)	(40.00***)	(22.00***)	(−7.14)	(−30.20***)
3235/8790	25.07***	40.92***	95.87***	−28.05***	24.14***	−9.33**	−14.02	−24.29**
	(12.66***)	(33.14***)	(76.00***)	(−37.68***)	(10.77***)	(−18.40***)	(−20.69**)	(−36.73***)
227/390	125.53***	140.65***	82.95***	141.76***	17.12***	83.33***	118.75***	−11.63
	(75.07***)	(100.36***)	(44.53***)	(71.61***)	(−4.41*)	(83.33***)	(105.88***)	(−13.58)
227/6554	74.83***	65.13***	59.85***	133.38***	−26.32***	75***	62.22***	29.59**
	(34.99***)	(31.92***)	(56.08***)	(57.30***)	(−38.24***)	(40.00***)	(21.67**)	(6.79)
227/411	196.73***	169.54***	151.82***	188.33***	−11.72***	95.56***	131.71***	−26.63***
	(165.20***)	(144.97***)	(128.63***)	(113.26***)	(−16.91***)	(46.67***)	(82.69***)	(−38.98***)
227/3214	−15.06***	−0.78	159.4***	−24.49***	−10.57***	36.25***	11.63	−19.47***
	(−33.45***)	(−21.49*)	(131.95***)	(−46.65***)	(−19.12***)	(9.00**)	(−14.29)	(−26.14***)
227/8790	59.28***	69.96***	64.36***	38.44***	12.78***	72.97***	79.55***	22.42**
	(15.59***)	(28.37***)	(61.08***)	(−12.86***)	(10.29***)	(28.00***)	(36.21***)	(10.29)
101/390	−9.63**	9.09	−12.14***	−18.92***	47.37***	21.83***	68.81***	89.21***
	(−16.29***)	(9.00)	(−26.96***)	(−28.51***)	(34.62***)	(−12.41***)	(22.67***)	(63.34***)
101/6554	16.49***	22.57*	39.89***	11.82**	36.73***	−17.3***	6.67	43.01**
	(7.17*)	(16.46)	(34.17***)	(−8.69*)	(28.85***)	(−28.47***)	(−4.00)	(38.24**)
101/411	103.26***	110.16***	121.93***	78.45***	10.71***	−5.06*	−25.98***	−49.56***
	(85.24***)	(90.43***)	(90.18***)	(67.01***)	(3.33)	(−10.95***)	(−37.33***)	(−63.16***)
101/3214	12.92***	40.67***	42.92***	6.63	68.22***	35.86***	−9.92	−23.26**
	(5.87*)	(32.03***)	(35.84***)	(−6.63)	(63.64***)	(17.52***)	(−21.33***)	(−39.40***)
101/8790	26.25***	37.31***	48.69***	28.28***	−31.62***	−23.66***	−39.85***	8.01
	(7.11*)	(21.44***)	(42.11***)	(−5.66*)	(−38.46***)	(−27.01***)	(−46.67***)	(−15.92*)
Mean	46.92	54.12	71.46	33.39	19.6	46.84	36.73	6.66
	(30.85)	(39.33)	(55.53)	(8.57)	(10.73)	(26.87)	(16.12)	(−11.03)

*Significant at $P < 0.05$

**Significant at $P < 0.01$

***Significant at $P < 0.001$

HPH in all eight root traits, indicating that this hybrid was the best cross for root heterosis.

Four differential expression patterns were found between hybrids and their parents

From 916 to 1042 fragments were displayed from each hybrid and its parents, with an average repeatability of 79.97% (from 74 to 86%) in the duplicated PCR (Wang et al. 2003). For each hybrid in the NC Design II mating scheme cross, both quantitative and qualitative differences in the displayed cDNA fragment patterns were observed. Since the quantitative differences could not be accurately examined, only the qualitative differences were considered in the following analysis. Qualitatively differentially expressed patterns fell into four different categories as we previously described (Sun et al. 2004): (i) fragments observed in both parents but not in F_1 ($BPnF_1$); (ii) fragments that occurred in one parent but not in another parent and F_1 ($UpnF_1$); (iii) fragments observed only in F_1 but not in either of the parents (F_1nBP); (iv) fragments present in one parent and F_1 but not in another parent (UPF_1). When analyzed across the 20 hybrids at jointing stage, $BPnF_1$ pattern accounted for 6.74% of the bands, $UPnF_1$ pattern for 5.92%, F_1nBP pattern for 4.38%, UPF_1 pattern for 10.48%, respectively, giving a total of 27.52% differentially expressed fragments of the bands (Table 3).

We further examined the variations for a given expression pattern in different hybrids. As shown in Table 3, for each patterns, variations exist among 20 hybrids. The percentage of $BPnF_1$ pattern ranged from 2.53 to 10.11%, $UPnF_1$ from 4.62 to 7.70%, F_1nBP from 1.26 to 6.64%. UPF_1 ranged from 7.34 to 14.91%.

Reverse-northern blot confirmation of differentially expressed cDNA fragments

Thirty cDNA fragments that are differentially expressed between a root heterotic hybrid (3338/6554) and its parental inbreds in duplicated DDRT were validated by reverse-northern blot (Fig. 1). Among the 30 cDNA fragments, nine (W27, W45, W46, W53, A1, A7, A8, A9, A10) were up-regulated in the hybrid and only one (W47) was down-regulated. Seventeen cDNA fragments (W1, W20, W29, W31, W36, W39, W41, W43, W51, W52, W57, K3, K6, K9, K10, K13, 10) were expressed at the same level in the hybrid as one parent. The expression amount of W49 was lower than one parent and higher than the other, whereas W34 was expressed in only one of the parents. K14 was expressed in the hybrid at the same level as one parent, but was not detected in the other parent (Table 4).

Characterization of differentially expressed genes

Most of the 30 differentially expressed cDNA clones that were sequenced have high similarity to cereal genes (Table 4). BLAST analysis indicated that the putative functions of these 30 differentially expressed genes were grouped into five classes. The first group includes those clones that had high similarity to wheat (*Triticum aestivum*) genes, including A1 (fructose 1, 6-biphosphorate aldolase) (100%), K6 (18S ribosomal RNA gene) (99%), K9 (*T. aestivum* mitochondrial DNA) (98%), W39 (DNA-binding protein) (89%), W41 (fructose 1, 6-biphosphorate aldolase) (96%) and A9 (PBF of Dof DNA-binding protein family) (74%). The second group includes clones that had high similarity to genes in *Hordeum vulgare*, *Triticum monococcum* and *Triticum turgidum*, including W20 (RNA-directed RNA polymerase 2) (89%), W46 [protein synthesis elongation factor-1 alpha (EF-1)] (96%), W49 (*T. monococcum* actin gene) (90%), W51 (*T. turgidum* A genome HMW glutenin A gene locus) (93%), W52 (putative cellulose synthase catalytic subunit) (97%) and A10 (phytochelatin synthetase) (98%). The third group includes those clones with high identities with genes in *Oryza sativa*, including W31 (NADH dehydrogenase subunit 5) (100%), W34 (putative avr9

Table 3 Percentage of each differential expression pattern in 20 crosses at jointing stage

Crosses	$BPnF_1$ (%)	$UPnF_1$ (%)	F_1nBP (%)	UPF_1 (%)	Total (%)
3338/390	6.26	5.70	4.31	12.10	28.37
3338/6554	9.24	5.96	4.02	11.33	30.55
3338/411	6.45	5.10	5.55	9.60	26.70
3338/3214	9.94	6.27	5.35	10.86	32.42
3338/8790	8.57	6.32	5.71	9.02	29.62
3235/390	2.53	5.90	1.26	11.80	21.49
3235/6554	4.02	4.72	2.22	8.60	19.56
3235/411	6.51	5.14	3.33	8.77	23.75
3235/3214	7.46	6.72	2.54	9.85	26.57
3235/8790	6.53	6.25	4.12	7.39	24.29
227/390	4.35	4.62	6.33	14.91	30.21
227/6554	3.46	7.35	3.17	10.66	24.64
227/411	5.30	5.30	3.94	9.55	24.09
227/3214	6.75	4.99	5.58	7.34	24.66
227/8790	4.76	5.05	6.64	9.81	26.26
101/390	9.10	5.88	5.74	10.92	31.64
101/6554	8.81	7.60	2.43	11.70	30.54
101/411	7.70	7.71	5.03	11.16	31.60
101/3214	7.01	5.05	4.49	12.20	28.75
101/8790	10.11	6.94	5.88	12.07	35.00
Mean	6.74	5.92	4.38	10.48	27.52

$BPnF_1$, bands observed in *B*oth *P*arents but *n*ot in the *F_1*; *$UPnF_1$*, bands occurring in only *U*ni-*P*arent but *n*ot in the *F_1* and another parent; *F_1nBP*, bands detected in only the *F_1* but *n*ot in *B*oth *P*arents; *UPF_1*, bands present in *U*ni-*P*arent and *F_1* but absent in another parent

Table 4 Expression patterns and sequence similarity of differentially expressed cDNA fragments

Clone name	DDRT expression patterns	Reverse-northern blot validation expression patterns	Best homology	Accession no.	Sequence identity (%)	*E* value
W20	UPnF_1	HOP	RNA-directed RNA polymerase, *H. vulgare*	gi40794967	89	3e−61
W29	F_1nBP	HOP	Unknown protein, *O. sativa*	gi41053211	53	3e−19
W31	UPF_1	HOP	NADH dehydrogenase subunit 5, *O. sativa*	gi74100122	100	4e−52
W34	UPF_1	UPnF_1	Putative avr9 elicitor response protein	gi50909623	74	2e−17
W36	UPF_1	HOP	Clathrin heavy chain, *O. sativa*	gi77548264	96	7e−49
W39	UPF_1	HOP	DNA-binding protein, *T. aestivum*	gi6958202	89	1e−71
W41	F_1nBP	HOP	Fructose 1,6-biphosphorate aldolase, *T. aestivum*	gi18496065	95	7e−66
W43	UPF_1	HOP	Putative splicing regulatory protein, *O. sativa*	gi18071362	85	1e−73
W46	F_1nBP	HH	Protein synthesis elongation factor-1 alpha, *H. vulgare*	gi396134	96	6e−80
W49	UPF_1	HOPLA	*T. monococcum* actin gene	gi15088543	90[a]	5e−108
W47	UPnF_1	HL	Retrotransposon protein, *O. sativa*	gi77554634	82	5e−48
W51	UPF_1	HOP	*T. turgidum* A genome HMW glutenin A gene locus	gi37575357	93[a]	1e−125
W52	UPnF_1	HOP	Putative cellulose synthase catalytic subunit, *H. vulgare*	gi39726029	97	2e−112
W53	F_1nBP	HH	MDR-like ABC transporter, *O. sativa*	gi50932093	75	2e−51
K6	UPF_1	HOP	18S ribosomal RNA gene, *T. aestivum*	gi15982656	99[a]	0.0
K9	F_1nBP	HOP	*T. aestivum* mitochondrial DNA	gi78675232	98[a]	0.0
K10	UPnF_1	HOP	OsJNBb0072M01.18, *O. sativa*	gi50926468	85	1e−59
K13	UPF_1	HOP	*O. sativa* chromosome 10 clone nbxb0018F16	gi21263268	82[a]	2e−24
A1	F_1nBP	HH	Fructose 1,6-biphosphorate aldolase, *T. aestivum*	gi18496065	100	5e−63
A7	F_1nBP	HH	Annexin, *O. sativa*	gi34905010	72	2e−32
A8	F_1nBP	HH	Extensin, *O. sativa*	gi34912294	80	1e−18
A9	F_1nBP	HH	DNA-binding protein(PBF), *T. aestivum*	gi3790264	99	5e−31
A10	F_1nBP	HH	Phytochelatin synthetase, *T. monococcum*	gi30090032	98	1e−76
W1	UPnF_1	HOP	No hit			
W27	BPnF_1	HH	No hit			
W45	F_1nBP	HH	No hit			
W57	BPnF_1	HOP	No hit			
K3	UPF_1	HOP	*T. aestivum* cDNA clone TaE05024F11F	gi20432813	93[a]	2e−41
K14	UPF_1	UPF_1	*T. aestivum* cDNA clone whyd6e21 5′	gi20112580	91[a]	3e−118
10	F_1nBP	HOP	*T. aestivum* cDNA clone rwhec3f23 3′	gi93084340	99[a]	4e−145

BPnF₁, bands observed in *B*oth *P*arents but *n*ot in the F_1; UPnF_1, bands/signals occurring in only *U*ni-*P*arent but *n*ot in the F_1 and another parent; *F_1nBP*, bands detected in only the F_1 but *n*ot in *B*oth *P*arents; *UPF_1*, bands present in *U*ni-*P*arent and F_1 but absent in another parent; *HL*, signals observed in both parents and hybrid F_1, but the amount of expression in *Hybrid* F_1 is *Lower* than its parents; *HH*, signals observed in both parents and hybrid F_1, but the amount of expression in *Hybrid* F_1 is *Higher* than its parents; *HOP*, signals observed in both parents and hybrid F_1, but the amount of expression in *Hybrid* F_1 was the same as *One Parent*; *HOPLA*, signals observed in both parents and hybrid F_1, but the amount of expression in hybrid F_1 was *Higher* than *One Parent* and *Lower* than *Another* parent

DDRT differential display reverse transcription

[a] Indicates nucleotide identity, while others are amino acid identities

elicitor response protein) (74%), W36 (putative clathrin heavy chain) (96%), W43 (putative splicing regulatory protein) (85%), W47 (retrotransposon protein) (82%), W53 (MDR-like ABC transporter) (75%), A7 (annexin) (72%), A8 (extensin) (84%), K10 (OsJNBb 0072m01.18) (85%), K13 (*O. sativa* chromosome 10 clone nbxb0018F16) (82%) and W29 (unknown protein) (53%). The fourth group includes those clones with high similarity to *T. aestivum* cDNA clone from wEST database, including K3 (TaE05024F11F) (93%), K14 (whyd6e21 5′) (91%) and 10 (rwhec3f23 3′) (99%). The fifth group includes those clones with no similarity to known genes and ESTs, including W1, W27, W45 and W57 (Table 4).

Isolation of three differentially expressed genes with ORFs

To test whether differentially expressed cDNA fragments truly represent the putative genes and provide a

basis for further analysis of their functions, the complete ORFs of three up-regulated cDNA fragments were assembled using in silico cloning and their expression patterns were determined by semi-quantitative RT-PCR.

Annexin plays an important role in root morphogenesis. The deduced amino acid sequence of a cDNA fragment (A7) shared considerable homology to a group of annexin proteins, with similarity ranged from 43 to 72%. A subsequent search of public database with the A7 sequence identified three overlapping wheat ESTs (gi9742164, gi20103388, gi20048060) that can be assembled into one sequence which contains complete ORF, designated *TaANN1*. To verify the results of the in silico cloning, gene-specific primers were designed to amplify the corresponding cDNA sequence in root. The nucleotide sequence of *TaAnn1* (GenBank accession no. AY462115) and its deduced amino acid sequence are shown in the supplementary Fig. 1. The 951-bp fragment contains an ORF encoding 316 amino acids, which begins with an ATG initiation codon at nucleotide 1–3 and ends with a TGA stop codon at nucleotide 949–951. The ORF specifies a protein with a predicted molecular mass of 35, 380 Da. The predicted protein of *TaANN1* had four repeat domains of the typical structure of annexin, with a potential heme-binding domain, the critical His residue needed for heme-binding and Ca^{2+}-binding sites consisted of the sequence GXGTD, which is followed by 42 amino acids downstream of the first Gly residue by a Glu. It also had other potentially important motifs, includes the GTP-binding motif (GXXXXGKT) and an F-actin-binding motif (IRI).

Plant gene expression involves classes of transcription factors that have specifically evolved to regulate plant-specific genes and/or to mediate a variety of plant-specific signals. The Dof (*D*NA-binding with *o*ne *f*inger) family is a typical example of such transcription factors (Yanagisawa 2002). BLASTX search revealed that A9, a 391-bp cDNA fragment that was up-regulated in the hybrid, has very high similarity (99%) to a subclass as the PBF of Dof proteins. With in silico cloning starting from the A9 cDNA sequence, an ORF was assembled from three overlapping dbEST sequences (gi20436734, gi25429824, gi20433676). An approximately 1 kb PCR product, termed as *TaPBF2*, was obtained with primers derived from the above predicted N-terminal (sense) and C-terminal (antisense) sequences. Sequencing confirmed the isolation of *TaPBF2* spanning the whole translation region of a Dof protein with a clear homology to PBF of Dof binding protein sequence, specifying a protein with a predicted molecular mass of 33, 958 Da (see supplement Fig. 2) (GenBank accession no. AY496057). The highly conserved Dof domain (residues 38–87) of *TaPBF2* has 100% identity to the corresponding region of five previously reported DNA-binding proteins, such as *HvB-PBF* (Mena et al. 2002). It was worthy to note that a structure of four cysteine residues putatively involved in Zn^{2+} co-ordination corresponding to a CX_2C—CX_2C zinc finger motif (residues 38–66) was present in the Dof region.

Schindelman et al. (2001) reported that COB was a regulator of oriented cell expansion, while Leuchter et al. (1998) demonstrated that partial COB cDNA can complement a *Saccharomyces pombe* mutant defective in phytochelatin synthesis. Li et al. (2003) reported that BC1, which encodes a COBRA-like protein, was found to play roles in the cell wall biosynthesis of rice. We obtained an over-expressed cDNA fragment in hybrid (A10), which had high degree of identity with both GPI-anchored protein COB (86%) and phytochelatin synthetase (98%). By using A10 as seed sequence, a complete ORF was obtained by assembling three ESTs (gi21481922, gi32775210, gi39000899). PCR amplification was performed using gene-specific primer, and yielded a distinct 1,571 bp product (Designated *TaCOB1* hereafter). Cloning and sequencing of *TaCOB1* cDNA revealed that it encodes a protein of 456aa with a predicted molecular mass of 50, 591 Da

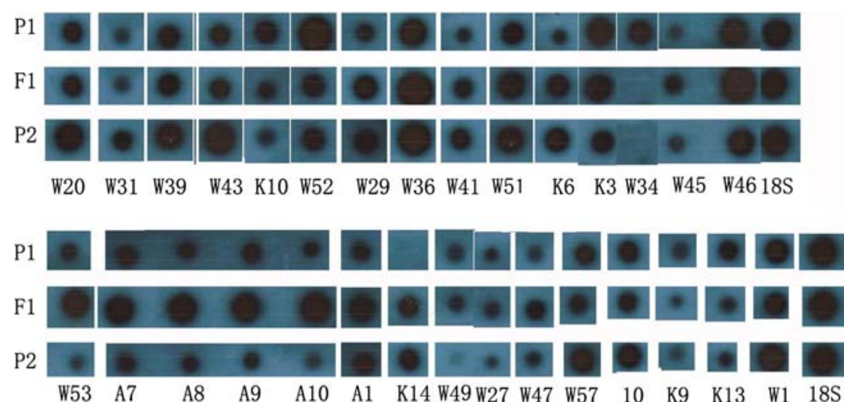

Fig. 1 Reverse-northern dot blot validation of the DDRT-detected differentially expressed cDNA fragments. P1—paternal parent (6554); F_1—hybrid (3338/6554); P2—maternal parent (3338). Product amplified by 18S primers used as control

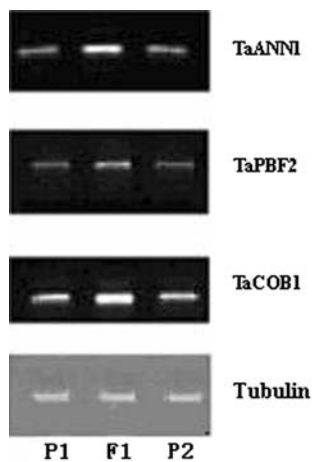

Fig. 2 Semi-quantitative RT-PCR analysis of *TaANN1*, *TaPBF2* and *TaCOB1*. The expression of tubulin gene served as an internal control: P1, 3338; P2, 6554; F_1, hybrid 3338/6554

(see supplement Fig. 3) (GenBank accession no. AY442329). This predicted ORF comprised of a putative cleavable N-terminal signal sequence (as determined with *pSORT*) and a GPI linkage motif including the predicted cleavage site, followed by a six amino acid spacer region. Furthermore, it also had potential C-terminal GPI-modification omega sites.

Semi-quantitative RT-PCR analysis showed that *TaANN1*, *TaPBF2* and *TaCOB1* were up-regulated in hybrid, which provides further support for the reverse-northern dot blot analysis of their expression patterns (Fig. 2).

Discussion

Heterosis in wheat root traits

To our knowledge, the study represents the largest and most systematic survey of heterosis in root traits hitherto reported in wheat. The results indicated that significant heterosis in wheat root traits can be observed, and many heterotic hybrids were identified for further analysis. Systematic survey for heterosis of root characters has been performed in *Arabidopsis* (Meyer et al. 2004), and significant heterosis for root mass was observed, with MPH of 56.9%. In the present study we found substantially higher levels of heterosis in wheat root traits in F_1 hybrids. Twenty-one out of 160 hybrid–trait combinations (13.13%) shown greater than 100% for MPH and 10 (6.25%) greater than 100% for HPH. In our previous study to investigate heterosis in aboveground agronomic traits, the highest MPH value of 30.83% was observed for grain yield per plant (Wu et al. 2001), while in the present study using the same crosses, the highest MPH value of 196.73% in root traits was observed for TRL. The mean MPH among 20 hybrids in six root traits was greater than 30% while the mean MPH in nine agronomic traits are below 13%. Therefore, it seems that substantially higher levels of heterosis are present in root traits than in aboveground agronomic traits. Liu et al. (1992) found that wheat hybrids were more stable in yield performance than parental pure lines, suggesting that hybrids were generally superior in buffering ability. Heterosis of root traits may contribute to this buffering ability.

Genes up-regulated in hybrid

Of the 30 cDNA clones, 9 (30%) were found to be over-expressed in hybrids relative to their parents. These results may be interpreted as molecular evidence for true heterosis in which it is predicted that the hybrid should have more abundant gene expression, which might contribute hybrid vigor.

Among the nine up-regulated cDNA fragments, W46 encoded protein synthesis EF-1, which plays a key role in protein synthesis. A1 encoded fructose 1, 6-biphosphorate aldolase, which catalyses the reversible aldol condensation of dihydroxy-acetone phosphate and glyceraldehydes-3-phosphate in the Calvin cycle, glycolysis and gluconeogenesis, and is thus essential for primary metabolism in all cells (Plaumann et al. 1997). Enhanced expression of A1 in hybrid might favor more energy production needed in hybrid. We also cloned and characterized an ORF of wheat PBF gene (A9), designated as *TaPBF2*. In barley, analysis revealed that PBF acts as an activator of reserve protein genes in the developing barley endosperm (Mena et al. 1998), or as a transcriptional repressor upon germination (Mena et al. 2002). Isolation of the wheat PBF gene argues for a general conservation of this class of Dof DNA-binding proteins as important regulators of storage protein gene expression across a wide spectrum of cereal crops (Mena et al. 1998). Recently, Diaz et al. (2002) reported that *HvGAMYB* protein interacted in developing barley endosperms with PBF, a Dof transcription activator of the *Hor2* gene. In our study, *TaPBF2* gene was up-regulated in hybrid root in wheat, suggesting that Dof transcript factor might have additional function in root development.

Interestingly, four up-regulated cDNA fragments (A7, A8, A10 and W53) in hybrid corresponded to genes involved in root morphogenesis. A7 was homologous to annexin gene. Annexin is a diverse, multigene family of calcium-dependent, membrane-binding proteins that serve as targets for Ca^{2+} in most eukaryotic

cells. The expression of plant annexin genes is influenced by environmental and developmental signals. For example, annexin mRNA levels are up-regulated in response to stress and abscisic acid in alfalfa (Kovacs et al. 1998). In situ hybridization experiments have identified that the maize p35 annexin is expressed in root cells and differentiating vascular tissue of roots (Carroll et al. 1998). Clark et al. (2001) proposed that *AnnAt1* is involved in the directed secretion of polysaccharides by root-cap cells and polar growth of root hair cells. The wheat annexin cloned in this study was up-regulated in hybrids, suggesting that hybrids may have higher ion channel activity than their parents, which could contribute to the enhanced root development.

The deduced amino acid sequence of A8 was found to be homologous to extensin proteins, such as *AtExt1* and *HRGPnt3*. Recently, progress has been made on the functional analysis of extensin genes. Sequence analysis showed that extensin protein had a highly repetitive Ser-$(Hyp)_4$ motif, in which the proline residues are firstly hydroxylated and then glycosylated (Merkouropoulos et al. 1999). It is synthesized as soluble protein, which subsequently becomes insolubilized in the plant cell wall through the intermolecular cross-linking of tyrosines on adjacent monomers (Kieliszewski and Lanport 1994). Study on the tobacco extension gene, *HRGPnt3*, showed that it was expressed in a subset of cells involved in lateral root initiation, thereby allowing root tips to withstand mechanical pressures arising from penetration of the lateral root through the primary root (Keller and Lamb 1989). In this study, the expression level of A8 in hybrid was higher than that of its two parents. Since extensins play a structural role in the cell wall, the putative function of A8 gene might be to increase the mechanical strength of the cell wall, which may in turn benefit hybrid root growth.

A10 had high similarity with both phytochelatin synthetase and GPI-anchored protein (COB). COBRA belongs to a multigene family consisting of 12 members in *Arabidopsis*, all of which are predicted to encode putative GPI-anchored proteins; they are designated COBRA-like proteins (Roudier et al. 2002). The COB protein was detected predominantly on the longitudinal sides of root cells in the zone of rapid elongation and RNA levels are dramatically up-regulated in cells entering the zone of rapid elongation. Based on these results, models were proposed for the role of COB as a regulator of oriented cell expansion (Schindelman et al. 2001). Although the *cob* mutant phenotype is most obvious in the epidermal cell layer, where the cell volume is approximately the same as wild type, cells in the cortex and endodermis also expand more radically and less longitudinally than in wild type (Hauser et al. 1995). In this study, since we found that *TaCOB1* was up-regulated in hybrid, we proposed that *TaCOB1* gene should play important roles for root heterosis. Furthermore, isolation of a partial *COB* cDNA was reported to complement a *S. pombe* mutant defective in phytochelatin synthesis (Leuchter et al. 1998). These are necessary to protect cells from divalent heavy metals, such as cadmium. The partial *COB* cDNA, which was able to confer resistance to cadmium in *S. pombe*, did not contain the first 94 amino acids of COB. These missing amino acids contain the putative N-terminal signal sequence as well as the domain in which the *cob-3* mutation is found (Schindelman et al. 2001). We also found *Escherichia coli* expressing *TaCOB1* significantly improved resistance to cadmium (our unpublished data). Although this reported phytochelatin synthesis activity may not be directly related to COB's *in planta* function, it is possible that COB binds divalent metals, and this feature could have function redundancy.

W53 showed high amino acid similarity (86%) to the MRP (Multidrug Resistance-associated Protein) family of ABC transporters from *Arabidopsis*. It has been suggested that MRP-like proteins such as *AtMRP5* could act as transporters for auxin conjugates such as the negatively charged indole acetic acid (IAA)-aspartate or IAA-glutamate (Ostin et al. 1998; Tam et al. 2000). Gaedeke et al. (2001) found that mrp5-1 mutant plants showed a strongly reduced root elongation associated with an earlier initiation of lateral root formation. Up-regulated expression of W53 in hybrid may create more efficient, NPA-sensitive auxin efflux mechanisms together with ion channels, thus contribute to the root heterosis.

Genes down-regulated in hybrid

Surprisingly, one selected cDNA clone (W47) had lower expression level in hybrid than either of its parents. Homology research showed that W47 is similar to a rice retrotransposon.

Parental preference in F_1 hybrid gene expression

In this study, half of the differentially expressed cDNA fragments (17 out of 30) observed in the hybrid were similar in hybridizing signal intensity to that of one parent. The seventeen cDNA fragments were W1, W20, W29, W31, W36, W39, W41, W43, W51, W52, W57, K3, K6, K9, K10, K13 and 10. On of these cDNA fragments, W52, had high amino acid similarity (92%) to a cellulose synthase from *Arabidopsis*, which may take a

role in root development. Cellulose forms semi-crystalline microfibrils, which impart considerable mechanical strength to roots (Reiter 1998). *Arabidopsis* KOJAK encodes a cellulose synthase-like protein, which is involved in the biosynthesis of beta-glucan-containing polysaccharides that are required for root hair cell morphogenesis during root hair elongation (Favery et al. 2001). Different CesA isoforms interact to form a functional cellulose synthase enzyme (Perrin 2001). Perhaps multiple isoforms of cellulose synthase are needed in the same cell for the formation of functional dimeric complexes.

Another clone, W31, which is similar to NADH dehydrogenase, is worth noting here because it was also found to be differentially expressed in wheat leaf tissue in the same wheat hybrid (Wu et al. 2003). It is thought that NADH dehydrogenases oxidize cytosolic and matrix NADH (Cook-Johnson et al. 1999). Little is known about the physiological role of the inner-membrane NAD (P) H dehydrogenases. They do not contribute to the production of ATP at site 1 and so the free energy released during electron transfer is lost as heat. Consequently, these enzymes represent a potentially energy wasteful system. It has been suggested that these enzymes may have a role in stress response. NADH dehydrogenase gene tends to be expressed at lower level in hybrid, which might suggest that the expression pattern of the gene could play a role in saving energy in hybrids.

In conclusion, this study demonstrated that root system traits of wheat F_1 hybrids shown significant positive heterosis, and up to 27% of genes were found to be differentially expressed between hybrids and their parents by using DDRT PCR analysis. Some of the differentially expressed genes are related to root development. We concluded that differential gene expression could play a role in root heterosis of wheat, and possible other cereal crops.

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