Identification of differentially expressed transcripts at critical developmental stages in sorghum [*Sorghum bicolor* (L.) Moench] in relation to grain yield heterosis

I. Jaikishan¹ · P. Rajendrakumar¹ · K. Hariprasanna¹ · D. Balakrishna¹ · B. Venkatesh Bhat¹ · Vilas A. Tonapi¹

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Abstract

Evaluation of a set of 10 F_1 hybrids along with their female (27A and 7A) and male parents (C 43, RS 673, RS 627, CB 26, and CB 29) for grain yield and its component traits revealed that grain yield/plant followed by panicle weight, primary branches/panicle, and 100-seed weight exhibited high levels of heterosis. Eight hybrids exhibited 50% or more mid-parent heterosis for grain yield/plant, of which, one hybrid (27A × RS673) recorded heterobeltiosis above 50% (73.61%). Differential display analysis generated about 2995 reproducible transcripts, which were categorized as UPF₁—expressed in any one of the parents and F_1 (10.53–14.76%), BPnF₁—expressed in both parents but not in F_1 (4.56–11.44%), UPnF₁—expressed in either of the parents and not in F_1 (17.95–27.40%), F_1 nBP—expressed only in F_1 but not in either of the parents (14.39–20.54%), and UET—expressed in both parents and F_1 (34.52–42.43%). A comparison between high and low heterotic hybrids revealed that the proportions of UPF₁ and F_1 nBP transcript patterns were much higher in the former (21.31% and 45.24%) as compared to the latter (16.67% and 32.14%) at the booting and flowering stage, respectively, indicating the role of over-dominance and dominance in the manifestation of grain yield heterosis. Significant positive correlations were observed for differential transcript patterns with mid-parent and better-parent heterosis for the components of grain yield such as primary branches (0.63 and 0.61 at $p < 0.01$) and 100-seed weight (0.64 and 0.52 at $p < 0.01$). Cloning and sequence analysis of 16 transcripts that were differentially expressed in hybrids and their parental lines revealed that they code for genes involved in basic cellular processes, cellulose biosynthesis, and assimilate partitioning between various organs and allocation between various pathways, pyrimidine, and polyamine biosynthesis, enhancing ATP production and regulation of plant growth and development.

Keywords Sorghum · Grain yield heterosis · Differential display analysis · Transcripts · Differential expression · Correlation

Introduction

Sorghum [*Sorghum bicolor* (L.) Moench], one of the important cereal crops, is traditionally grown as food as well as fodder by resource poor farmers with minimum inputs in the semi-arid tropical regions of the world. Globally, sorghum is cultivated on 44.7 m ha accounting for the production of

63.9 m tons. In India, the area occupied by this crop was around 5.65 m ha in 2016 generating a total production of 4.4 m tons (FAO 2017). The crop is cultivated both in rainy (*kharif*) and post-rainy (*rabi*) seasons with the predominance of hybrids and open pollinated varieties, respectively (Sanjana Reddy 2018). It has emerged as a potential alternative feed (Dahlberg et al. 2015) and bio-energy crop (Bellmer et al. 2010) in recent times due to the rapid growth of poultry industry and growing demand for fuel grade ethanol. In addition, its resilience to high temperatures and drought makes it more relevant in the context of global climate change.

With the depleting land and water resources combined with the effects of climate change, hybrid sorghum is the most promising technology which has the potential to meet the future food, feed, and fodder production requirements across the world (Jaikishan et al. 2017). Even after

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✉ P. Rajendrakumar
rajendra@millets.res.in

¹ ICAR-Indian Institute of Millets Research, Hyderabad, Telangana 500030, India

successful exploitation of heterosis in sorghum for over six decades, the genetic components of heterosis are still not clear, the foremost being the number and nature of genes involved. However, the advent of molecular marker technology followed by the generation of high-density molecular linkage maps have assisted the researchers to dissect the genetic factors associated with the complex biological phenomenon of heterosis. Depending on the materials and experimental designs employed, variable results have been obtained from the genetic analysis of heterosis mostly in maize and rice based on molecular marker linkage maps (Chu et al. 2012; Xiang et al. 2016; Li et al. 2017). Since the beginning of twenty-first century, analysis of differential gene expression through various techniques has gained importance leading to the dissection of molecular mechanisms associated with plant development, disease response, heterosis, etc.

Heterosis for grain yield is mainly due to increased grain number per panicle (Blum 1970) and a larger panicle size (Miller and Kebede 1984). Grain yield is an outcome from several reproductive, morphological, and phenological characters such as plant height, panicle length, panicle weight, primary branches per panicle, test weight, etc. (Madhusudhana 2015). Sorghum hybrids exhibited a consistent yield increase (more than double) as compared to varieties under diverse growing and management conditions (Quinby 1958; Doggett 1988). Hence, it is important to dissect the factors contributing to the molecular basis of grain yield heterosis, which will be helpful in devising strategies for the development of superior high yielding hybrids. In the recent past, three heterotic trait loci (HTL) were identified in sorghum-exhibiting synergistic intra-locus effects on grain yield heterosis accounting for 19.0% of the heterotic variation (Ben-Israel et al. 2012). Non-availability of similar studies towards the identification of genes/loci associated with grain yield heterosis offers an excellent opportunity for the dissection of this complex biological phenomenon at the level of gene expression.

Investigations on heterosis in different crops have contributed to identification and characterization of differentially expressed genes (DEGs) related to heterosis (Zhang et al. 2008; Wei et al. 2009; He et al. 2010). The previous studies have indicated a correlation between the patterns of differential gene expression and heterosis (Xiong et al. 1998; Wu et al. 2001). Analysis of differential expression of genes at different developmental stages of hybrids and their parental lines have proven to be a useful methodology to identify the genes associated with heterosis. Among the various techniques applied to pin down genes involved in heterosis, differential display (Zhang et al. 2012), cDNA AFLP (Nie et al. 2015) and microarrays (Bassene et al. 2010) have been commonly used in various crop plants. The objectives of this study are to identify different transcript patterns in a

set of ten sorghum hybrids and their parental lines at three developmental stages, analyze the correlations of differential transcript patterns with grain yield heterosis, and determine the putative functions of selected transcripts through cloning and sequencing.

Materials and methods

Plant material

Two male sterile lines of A₁ cytoplasm (27A and 7A) were used to cross with five restorer lines (C 43, RS 627, RS 673, CB 26, and CB 29) in a factorial design to get 10 F₁ hybrids. These parental lines are adapted to rainy season. The pedigree and salient features of the parental lines are given in Table 1.

Estimation of grain yield heterosis

F₁ hybrids (10) along with the parental lines (7) were evaluated in a randomized complete block design with three replications during the rainy season of 2012 and 2013 in the Research Farm at the ICAR-Indian Institute of Millets Research, Hyderabad, India (latitude 17°19'N, longitude 78°23'E, 542.3 m above mean sea level). The crop was sown on 15 June and harvested during the third week of October. During this growth period, the average maximum temperature, minimum temperature, wind speed, and rainfall were 30.7 °C, 22.3 °C, 7.7 km h⁻¹, and 675 mm, respectively. The individual plot size was 3.6 m² (2 rows of 3 m length) and spacing was of 60 cm × 15 cm. About 80:40:40 kg NPK ha⁻¹ was applied in the field. Half dose of nitrogen (40 kg ha⁻¹), full dose of phosphorous (40 kg ha⁻¹ P₂O₅), and potash (40 kg ha⁻¹ K₂O) were applied during sowing, while the remaining dose of nitrogen was as applied as topdressing at 30 days after sowing. The recommended plant protection practices (spraying of cypermethrin 10EC @ 0.02% at 1 week after emergence of the crop and application of Carbofuran 3G @ 10 kg/ha in leaf whorls at 30 days after sowing) were followed to raise a healthy crop. Agro-morphological data for important agronomic traits like days to 50% flowering, plant height, panicle length, panicle weight, number of primary branches per panicle, grain yield per panicle, and 100-seed weight were recorded from ten plants per replication.

Differential display analysis

Description of growth stages in sorghum was first given by Vanderlip and Reeves (1972), which was simplified by Eastin (1972) into three growth stages viz., planting to panicle initiation (GS1), panicle initiation to flowering (GS2), and

Table 1 Plant material used in this study

Parents	Pedigree	Salient features
27A	Derived from the cross 83B × 199B	Tan colored plant with green, broad and erect leaves; medium thick stem with well exerted, semi-lax and cylindrical earhead; short glume; bold and round cream colored seed with light yellow and corneous endosperm
7A	Selection from AKMS 14A	Tan colored plant with pale green and drooping leaves; thick stem; exerted, semi-compact and elliptical earhead; straw colored glume without awns; freely threshable, medium size, corneous and round seed
C 43	Derived from the cross CS 3541 × IS 23549	Tan colored plant with green and semi-erect leaves; thick stem; compact and conical panicle with light red glume without awns; freely threshable grain; white, round, lustrous and bold seed with corneous endosperm
RS 627	Derived from the cross RS 71 × M 35-1	Tan colored plant with enclosed inter-node; green colored drooping leaves; elliptical and semi-compact panicle with short and straw colored glume; freely threshable, medium size round and white seed
RS 673	Derived from the cross SPV 544 × K 24-1	Tan colored plant with green and drooping leaves; medium thick stem; well exerted earhead; semi-compact and conical panicle with straw colored glume without awns; partial threshable grain; white, round, lustrous and medium bold seed with corneous endosperm
CB 26	Derived from the cross SB 1066 × SPV 775	Tan colored plant with drooping leaves; semi-compact panicle; straw colored glume; white, round and bold seed
CB 29	Derived from the cross NSV 13 × SPV 475	Tan colored plant; semi-compact panicle with light red glume; white and easily threshable grains, which are white, bold and flat on one side

flowering to physiological maturity (GS3). In this study, the leaf samples were collected from seven parental lines and ten hybrids at vegetative, booting, and flowering stages, which represent the above-mentioned stages, and immediately frozen in liquid nitrogen. The top leaves of two plants per row of each genotype per replication devoid of any pest and disease damage were collected at the above-mentioned stages. The samples collected from three replications (two rows per replication) per genotype were pooled and used for extraction of total RNA. They were ground in liquid nitrogen and the total RNA was extracted using TRIzol (Invitrogen, USA) according to the manufacturer's protocol. The RNA quantification was done by Nanodrop Spectrophotometer which revealed good RNA quality at A260/A280 ratio of 1.9–2.0.

cDNA was synthesized using the Verso cDNA synthesis kit, which includes RT enhancer and RNase inhibitor (Thermo Fisher Scientific) as per manufacturer's instructions. Each 20 µl reaction mixture contained 3 µg total RNA, 50 mmol/L Tris-HCl (pH 8.3), 75 mmol/L KCl, 3 mmol/L MgCl₂, 10 mmol/L DTT (DTT, dithiothreitol), 50 mmol/L dNTPs, 50 pmol 3' end anchored primer, and 200 U M-MLV reverse transcriptase. The reaction mixture was incubated at 37 °C for 2 h. A set of 3' end anchored primers and 5' end oligonucleotide primers were used for differential display analysis as per von der Kammer et al. (1999), and were synthesized from Integrated DNA Technologies, USA. The details of oligonucleotide primers are given in Table 2.

The cDNA from the parental lines and 10 F₁ hybrids from three critical developmental stages from the samples collected were subjected to PCR amplification using the 3' end anchored primer and one of the 5' end oligonucleotide

Table 2 Oligonucleotide primers used in this study

Primer name	Primer sequence
3' end anchored primers	
W 01	5'-AAGCTTTTTTTTTTTTA-3'
W 03	5'-AAGCTTTTTTTTTTTC-3'
W 03	5'-AAGCTTTTTTTTTTTTG-3'
5' end oligonucleotide primers	
D 01	5'-TGCCGAAGCTTTGGTAGC-3'
D 02	5'-TGCCGAAGCTTTGGTCAC-3'
D 03	5'-TGCCGAAGCTTTGGTCAG-3'
D 04	5'-TGCCGAAGCTTTGGTCAT-3'
D 05	5'-TGCCGAAGCTTTGGTCGG-3'
D 06	5'-TGCCGAATTCTGGTCAC-3'
D 07	5'-TGCCGAATTCTGGTCAT-3'
D 08	5'-TGCCGAAGCTTGATTCCG-3'
D 08	5'-TGCCGAAGCTTGATTCCG-3'
D 10	5'-TGCCGAAGCTTTGGTCTG-3'

primer in separate reactions. The 25 µL reaction mixture comprised of 2 µL of cDNA, 2.5 µL of 10× reaction buffer, 150 µmol/L dNTPs, 5 pmol each of 3' end anchored primer, and 5' end oligonucleotide primer and 1 Unit of *Taq* DNA polymerase. The following thermal profile was followed for amplification: one cycle of initial denaturation at 94 °C for 5 min, pre-annealing at 40 °C for 4 min, pre-extension at 72 °C for 1 min, followed by 40 cycles of denaturation at 94 °C at 45 s, primer annealing at 60–62 °C for 1 min, and primer extension at 72 °C for 1 min. After the completion of 40 cycles, a final extension was followed at 72 °C for

10 min. Amplified products were resolved in 6% denaturing PAGE in Tris–borate buffer in a temperature controlled Bio-Rad Sequencing System at 50 °C followed by silver staining (Panaud et al. 1996).

Data analysis

All of the agro-morphological data collected from Randomized Blocks Design with three replications were subjected to analysis of variance (ANOVA) using the Statistix 8.1 software. Heterosis over mid-parent (mid-parent heterosis) and better-parent (heterobeltiosis) were estimated as per Alam et al. (2004):

$$\text{Mid-parent Heterosis}(H_{mp}) = [(X_x - X_{mp}) / X_{mp}] * 100$$

$$\text{Better parent Heterosis}(H_{bp}) = [(X_x - X_{bp}) / X_{bp}] * 100$$

where ' X_x ' is the observed mean value of the hybrid, ' X_{mp} ' is the mean of mid-parent value, and ' X_{bp} ' is the mean of better parent. The significance of heterosis values was tested by Student's t test with least significance difference (LSD) test at the probability level of 5% ($p \leq 0.05$) and 1% ($p \leq 0.01$) (Steel et al. 1997).

To eliminate false positives, the transcripts that were expressed in the samples collected during both the seasons (rainy seasons of 2012 and 2013) were considered for further analysis. All the differentially expressed cDNA fragments between 200 to 1000 bp were considered and were classified into four different categories of different gene-expression patterns (Fig. 1) as per Sun et al. (2004) (a) transcript expressed only in the hybrid, but not in both the parents (F_1nBP); (b) transcript expressed in any one of the parents and F_1 hybrid (UPF_1); (c) transcript expressed in both the parents, but not in F_1 hybrid ($BPnF_1$); and (d) transcript expressed in any one of the parents, but not in the other parent and F_1 hybrid ($UPnF_1$). The transcripts that were expressed in both parents and the F_1 hybrid were considered as universally expressed transcripts (UETs). The proportion of transcripts under each pattern was calculated for each

hybrid and their parental lines by calculating the percentage of the number of transcripts of a particular pattern over the total number of transcripts. The significance of the proportion of the transcripts across hybrids was tested by Student's t test with least significance difference (LSD) test at the probability level of 5% ($p \leq 0.05$) and 1% ($p \leq 0.01$) (Steel et al. 1997). Pearson correlation coefficients were calculated between different patterns of gene expression and grain yield heterosis (mid-parent and better-parent heterosis) at critical developmental stages using the Microsoft Excel software. The significance of correlation values was tested by t test.

Cloning and sequencing of differentially expressed transcripts

Transcripts that were differentially expressed between the hybrids and their parental lines were excised from the DD-PAGE gels and purified by dissolving the gel in 500 μ l of extraction buffer consisting of 500 mM ammonium acetate, 0.1% SDS, and 0.1 mM EDTA, followed by sodium acetate precipitation and 70% ethanol wash. The final pellet was dissolved in 50 μ l of nuclease free water.

The transcripts were re-amplified using the following thermal profile: initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 45 s, primer annealing at 60–62 °C for 1 min, and primer extension at 72 °C for 1 min and a final extension at 72 °C for 10 min. The amplified cDNA fragments were purified using GeneJET PCR Purification Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. The differentially expressed transcripts were inserted into TA-cloning vector pTZ57R/T and cloned in *Escherichia coli* using Gene-TA Cloning Kit (Genetix Biotech Asia) as per the manufacturer's protocol and then sequenced through Sanger Sequencing Technology by Integrated DNA Technologies, USA. Three clones of each transcript were sequence to get the consensus sequence. Homology search and annotations of the transcript sequences were performed using BLAST search against GenBank database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) with default algorithm parameters.

Fig. 1 Classification of differential expression patterns of transcripts. A, H, and R represent the female parent, F_1 hybrid, and the male parent, respectively. **a** F_1nBP —transcript expressed only in the hybrid, but not in both the parents; **b** UPF_1 —transcript expressed in any one

of the parents and F_1 hybrid; **c** $BPnF_1$ —transcript expressed in both the parents, but not in F_1 hybrid, and **d** $UPnF_1$ —transcript expressed in any one of the parents, but not in the other parent and F_1 hybrid

Results and discussion

Heterosis is being exploited commercially in sorghum as early as the 1960s resulting in the release of more than 30 hybrids for cultivation in India, predominantly for the rainy season. Even though an increase or decrease in estimates of mid-parent heterosis and heterobeltiosis indicates the true potential of hybrids (Hochholdinger and Hoecker 2007), the latter is considered as the more realistic and practical estimate, since it accounts for the performance of the hybrid against the best parent unlike the former that compares the hybrid against the mean of two parents (Lamkey and Edwards 1999). Moreover, selection of potential parents for the development of superior hybrids depends on effects of mid-parent heterosis and heterobeltiosis (Reif et al. 2007).

Estimation of grain yield heterosis

A wide range of mid-parent heterosis and heterobeltiosis was observed for grain yield/plant, panicle weight, primary branches/panicle, and 100-seed weight (Table 3) in the present study, which was similar to that reported in sorghum by various researchers (Haussmann et al. 2000; Hemlata

and Vital 2006; Ringo et al. 2015). A significant variation among the hybrids was observed for mid-parent heterosis [23.48 (7A × CB 26) to 97.22% (27A × RS 673)] and heterobeltiosis [5.57 (7A × CB 26) to 73.61% (27A × RS 673)] for grain yield, which indicated the presence of sufficient genetic divergence among the parental lines. Similar observation was also reported by Murty et al. (1994) and Ringo et al. (2015). Wide range of heterobeltiosis for grain yield/plant in sorghum was reported in the past by Elwafa et al. (2005). The extent of mid-parent heterosis in the present study was higher than 88% and 81.90% reported by Haussmann et al. (2000) and Ringo et al. (2015), respectively. Similarly, the heterobeltiosis was more than 69.52% and closer to 77.18% reported by Hemlata and Vital (2006) and Ringo et al. (2015), respectively. However, a similar higher heterobeltiosis for grain yield/plant of 103.09% had been reported by Hovny (2000). Panicle weight is an important component of grain yield, for which, the mid-parent heterosis ranged from 19.33% (7A × CB 26) to −85.92% (27A × RS 673), while the better-parent heterosis ranged between 2.45% (7A × CB 26) and 67.00% (27A × RS 673). Mid-parent and better-parent heterosis for 100-seed weight ranged from 2.86% (27A × CB 29) to 40.46% (27A × RS 673) and from −5.41% (27A × CB 29) to 22.30% (7A × RS

Table 3 Mid-parent and better-parent heterosis of agronomic important traits pooled over 2 years

Hybrids	Heterosis						
	Days to 50% flowering	Plant height	Panicle length	Panicle weight	Primary branches/panicle	Grain yield/plant	100-seed weight
27A × C43	−2.63 1.55**	26.99** 20.33**	20.04** 6.13	50.73** 34.06**	44.29** 38.28*	51.12** 31.18**	17.54* 6.22
27A × RS673	−4.99* −0.78**	31.91** 25.96*	19.93** 14.04**	85.92** 67.00**	42.53** 26.73*	97.22** 73.61**	40.46** 22.30*
27A × RS627	−3.81 5.18**	36.16** 27.45**	18.80** 8.76*	50.86* 37.35	11.27 3.93	60.29* 45.09	30.75 21.12
27A × CB26	−0.16 1.62	21.28* 11.18	12.47** 8.39**	56.46** 37.03	21.31 17.17	63.73** 42.18	12.50 9.25
27A × CB29	−2.28 1.85	26.74** 29.57*	15.16** 9.99**	56.76** 38.36**	29.69** 20.74*	63.78** 43.74**	2.86 −5.41
7A × C43	−7.70** 0.51**	39.13** 36.20**	18.73** 8.78**	46.63** 31.50**	11.86 8.94	52.89** 35.52**	16.81 6.24
7A × RS673	−8.52** 0.51**	20.03* 6.03	11.64* 7.20	54.26** 37.79**	2.17 −13.75**	60.88** 42.22**	31.38* 15.03
7A × RS627	−8.96** 2.23**	17.65 9.87	14.35 8.19	51.55** 35.03**	12.83 −0.79	59.84** 41.27**	16.40 8.43
7A × CB26	−7.49* −1.05**	16.04 −1.12	4.97 8.24	19.33** 2.45	−7.69 −15.89	23.48** 5.57**	6.95 5.14
7A × CB29	−11.88** −3.45**	24.64 11.71	11.16* 9.97*	39.82** 23.12**	16.33 2.09	45.96** 29.27**	22.14** 8.66

The values represented in * and ** are significant at $p < 0.05$ and at $p < 0.01$, respectively

Pooled over 2012 and 2013

Better-parent heterosis values are in bold

673), respectively. The maximum better-parent heterosis for grain yield/plant was exhibited by the hybrid 27A×RS 673 (73.61%), which also recorded the highest mid-parent heterosis (97.22%). Among the hybrids studied, six hybrids (27A×RS 673, 27A×RS 627, 27A×CB 26, 7A×RS 627, 7A×RS 673 and 27A×CB 29) recorded an estimated mid-parent heterosis of $\geq 50\%$ for grain yield/plant. Four of these hybrids (27A×RS 673, 27A×RS 627, 27A×CB 26 and 7A×RS 627) also recorded more than 50% better-parent heterosis (Table 3). Majority of the hybrids that exhibited positive and significant heterosis for grain yield/plant also showed similar trend for most of the component traits contributing to grain yield as reported by Hemlata and Vithal (2006), Jain and Patel (2013) and Ringo et al. (2015).

Patterns of differential gene expression

Analysis of differential gene-expression patterns in 10 sorghum hybrids and their corresponding inbred lines at three different developmental stages (vegetative, booting, and flowering stages) through differential display analysis resulted in the identification of differentially expressed transcripts, which followed both the qualitative and quantitative patterns as observed in the previous studies conducted in different crops (Sun et al. 2004; Meng et al. 2005). As the quantitative differential expression patterns were difficult to identify with accuracy, the analysis was done with qualitative differential patterns observed during the three developmental stages. To avoid false positives, the differential display analysis was performed using the total RNA extracted from the leaf samples of the sorghum hybrids and their parental lines during the rainy seasons of 2012 and 2013. Transcripts

that were amplified in both these samples were considered as positive ones. Analysis of differential expression patterns among the sorghum hybrids and their parental lines over 2 years revealed that 79% of the transcripts were reproducible, which is similar to the observations reported by Xing et al. (2006) and Zhao et al. (2009). The transcripts that are reproducible were considered for their classification into different expression patterns. A total of 2995 reproducible transcripts were identified from ten hybrids and their corresponding parental lines at three developmental stages with the nine primer combinations with a range of 253–336 transcripts per primer combination in the size range of 100–1000 bases. The differential expression patterns observed in this study were classified into four categories viz., F_1 nBP, UPF_1 , $BPnF_1$, and $UPnF_1$ patterns as per Sun et al. (2004). More than 60% of the genes were differentially expressed between ten hybrids and their corresponding inbred lines (Table 4) which was similar to those reported in maize (Meng et al. 2005) and in cotton (Zhao et al. 2009), where they found more than 50% of genes that were differentially expressed between hybrids and their parents.

During the three developmental stages, the proportion of differentially expressed transcripts ranged from 57.56% (27A×RS 673) to 65.48% (7A×RS 627). Analysis of the patterns of transcript expression revealed that the maximum proportion was accounted by $UPnF_1$ (21.58%) followed by F_1 nBP (18.39%), UPF_1 (12.74%), and $BPnF_1$ (7.68%) patterns, giving a total of 60.38% differentially expressed transcripts (Table 4). Variations in the proportion of different patterns of expression were also observed among the 10 hybrids at the critical developmental stages as revealed by the proportions of F_1 nBP (14.39–20.54%),

Table 4 Proportional distribution of differential expression patterns

Hybrids	Universally expressed transcripts (UETs)	Differentially expressed transcripts (DETs)				
		F_1 nBP (%)	UPF_1 (%)	$BPnF_1$ (%)	$UPnF_1$ (%)	Total
27A×C 43	39.48*	14.39**	14.76*	11.44**	19.93	60.52
27A×RS 673	42.43*	19.19	10.70*	9.23	18.45**	57.57
27A×RS 627	42.30*	17.39	13.04	7.51	19.76*	57.70
27A×CB 26	37.73	15.66*	14.23	4.98*	27.40**	62.27
27A×CB 29	38.09	19.78	14.29	9.89*	17.95**	61.91
7A×C 43	38.90	20.06	14.29	4.56**	22.19	61.10
7A×RS 673	38.14	18.92	12.61	8.41	21.92	61.86
7A×RS 627	34.52**	20.54*	12.20	6.55	26.19**	65.48
7A×CB 26	42.41*	18.58	10.53*	5.88*	22.60	57.59
7A×CB 29	42.16	19.38	10.77*	8.31	19.38*	57.84
Mean	39.61	18.39	12.74	7.68	21.58	60.39

The values represented in * and ** are significant at $p < 0.05$ and at $p < 0.01$, respectively

UET—universally expressed transcript (both parents and F_1 hybrid); F_1 nBP—transcript expressed only in the hybrid, but not in both the parents; UPF_1 —transcript expressed in any one of the parents and F_1 hybrid; $BPnF_1$ —transcript expressed in both the parents, but not in F_1 hybrid; and $UPnF_1$ —transcript expressed in any one of the parents, but not in the other parent and F_1 hybrid

UPF₁ (10.53–14.76%), BPnF₁ (4.56–11.44%), and UPnF₁ (17.95–27.40%) patterns. The distribution of differential gene-expression patterns in ten hybrids at three different growth stages is shown in Fig. 2 and the distribution of each differential gene-expression pattern between ten hybrids is shown in Supplementary Figs. 1–5. It is clear from the proportion of transcripts that the UPnF₁ pattern is the most prominent category of differentially expressed transcripts at all the three stages.

A substantial variation was observed in the four categories of differentially expressed transcripts among the ten crosses analyzed at three critical developmental stages, revealing the fact that the differences in transcript patterns may lead to differences in the degree of heterosis among various hybrids. The F₁nBP pattern was observed to be high in four hybrids (27A × C 43, 27A × CB 26, 27A × CB 29, and 7A × CB 29) during the booting stage, while in only two hybrids (27A × RS 673 and 7A × RS 627), the percentage of this pattern was high in flowering stage (Supplementary Fig. 1). Similarly, the percentage of BPnF₁ pattern in four hybrids (27A × C 43, 27A × RS 627, 27A × CB 29 and 7A × CB 29) was high at the flowering stage as compared to vegetative and booting stages (Supplementary Fig. 3). Seven hybrids recorded high percentage of UPnF₁ pattern at the vegetative stage, which gradually decreased at the flowering stage (Supplementary Fig. 4). Similar pattern of distribution of differentially expressed genes into four categories as mentioned above was observed in maize (Wang et al. 2007), wheat (Wang et al. 2006) and cotton (Zhao et al. 2009).

Fig. 2 Proportion of differential expression patterns at critical developmental stages. F₁nBP—transcript expressed only in the hybrid, but not in both the parents; UPF₁—transcript expressed in any one of the parents and F₁ hybrid; BPnF₁—transcript expressed in both the parents, but not in F₁ hybrid; UPnF₁—transcript expressed in any one of the parents, but not in the other parent and F₁ hybrid, and UET—universally expressed transcript (parents and F₁ hybrid)

Apart from the four categories of the differentially expressed transcripts mentioned above, a major proportion of the total transcripts expressed among the hybrids and their parents fall under the category of universally expressed transcripts (UET). About 39.61% of the total transcripts were expressed universally in hybrids as well as their parents (Table 4), which indicate that these may belong to housekeeping genes that are essential to sustain the metabolism and plant development and may be highly conserved during the course of evolution.

Comparison of differential expression patterns between high heterotic hybrid (27A × RS 673) and low heterotic hybrid (7A × CB 26) revealed some interesting observations (Supplementary Fig. 6). The percentage of F₁nBP pattern was high in the hybrid 7A × CB 26 as compared to 27A × RS 673 at both vegetative and booting stages, but the F₁nBP percentage is high in the hybrid 27A × RS 673 than 7A × CB 26 during the flowering stage. The percentage of UPF₁ pattern was almost similar in the hybrids 27A × RS 673 and 7A × CB 26 at vegetative and flowering stages, but this pattern was high in the hybrid 7A × CB 26 than 27A × RS 673 during the booting stage. The percentage of BPnF₁ pattern was high in the hybrid 27A × RS 673 in both vegetative and flowering stages than 7A × CB 26, but the percentage of this pattern was high in the hybrid 7A × CB 26 as compared to 27A × RS 673 during the booting stage. The percentage of UPnF₁ pattern was high in the hybrid 27A × RS 673 than 7A × CB 26 in the vegetative stage, but low in the hybrid 27A × RS 673 during booting and flowering stages than 7A × CB 26. When the overall percentages of the differential expression patterns were considered during all three developmental stages, the proportion of F₁nBP and UPF₁ patterns was almost similar in both the hybrids 27A × RS 673 and 7A × CB 26. The BPnF₁ pattern was higher in the hybrid 27A × RS 673 than 7A × CB 26, while the proportion of UPnF₁ pattern was higher in the hybrid 7A × CB 26 than 27A × RS 673.

While comparing high and low heterotic hybrids, the most significant changes in transcript patterns of UPF₁ and BPnF₁ were observed during the booting stage (Supplementary Fig. 6), which implies that the differential transcript pattern plays a vital role in yield and hybrid performance at this stage. Furthermore, the differences of transcript patterns between high and low heterotic hybrids revealed that the proportions of F₁nBP (over-dominant expression) and UPF₁ (dominant expression) patterns were much higher in the high heterotic hybrid as compared to the low heterotic hybrid at flowering and booting stage, respectively. As per the over-dominance hypothesis, the heterozygote of the locus is superior to either of the homozygotes, which would predict a positive correlation between hybrid-specific expression and heterosis. The present investigation also shows that the proportion of F₁nBP pattern at booting as well as flowering stages was higher in the high heterotic hybrid than the low

heterotic hybrid. This observation is in accordance with the reports of Sun et al. (2004) and Zhao et al. (2009), which concluded that genes specifically expressed in hybrids but not in both parents are highly correlated with heterosis.

Molecular characterization of differentially expressed transcripts

Sixteen transcripts that were differentially expressed in the hybrids and parental lines were extracted from the differential display gels, cloned, and sequenced (Table 5) revealed that the majority of these genes are involved in plant development, metabolism, transcriptional control, and signal transduction. These transcripts were submitted to GenBank with the Accession Numbers MG436937–MG436952. Among the 16 differentially expressed transcripts two transcripts, F 12 excised from 27B and F 30 excised from C 43 exhibited BPnF₁ pattern, one fragment (F 45) eluted from F₁ of 7A × RS 673 exhibited UPF₁ pattern, nine fragments eluted from F₁s (F 5, F 16, F 22, F 25, F 28, F 34, F 36, F 42, and F 49) showed F₁nBP pattern, one fragment F 10 eluted from CB 29 showed UPnF₁ pattern, and three fragments eluted from the parental line RS 627 (F 18) and F₁s (F 20 and F 46) exhibited UET pattern. The predicted product of the transcript F 42 (F₁nBP transcript pattern) exhibited 98% similarity to an NADP-dependent glyceraldehydes-3-phosphate dehydrogenase from sorghum (Sobic.007G140700.1). Glyceraldehyde-3-phosphate dehydrogenase is one of the crucial enzymes participating in the glycolytic pathway and the vital role of higher expression of this protein in sorghum–sudan grass hybrids in accelerating the pathway resulting in enhanced ATP production leading to the manifestation of heterosis has been reported (Han et al. 2016). The differentially expressed transcript F 5 (F₁nBP transcript pattern) showed 99% similarity to *Sorghum bicolor* UMP-CMP Kinase 4 (Sobic.007G223100.1), which is important for the de novo biosynthesis pyrimidine nucleotide, thereby catalyzing the phosphorylation of pyrimidine nucleoside monophosphates at the expense of ATP (Liljelund and Lacroute 1986). The transcript F 12 (BPnF₁ transcript pattern) eluted from CMS line 27A at booting stage exhibited 97% similarity to *Sorghum bicolor* two-component response regulator-like PRR73 (Sobic.001G411400.1), which is one of the components of circadian clock controlling the flowering response to photoperiod. Epigenetic modifications in circadian clock genes of maize and *Arabidopsis* and their altered expression were found to contribute to increased biomass leading to heterosis (Ni et al. 2009; Groszmann et al. 2011). The over-dominant gene action of a flowering gene in tomato has been found to contribute to yield heterosis (Krieger et al. 2010). The transcript F 16 (F₁nBP) was eluted from F₁s of different crosses,

which showed high similarity (99% homology) to a triose-phosphate translocator (TPT) (Sobic.003G002300.1), a chloroplast envelop membrane protein that is central for the communication between chloroplasts and cytosol. The simultaneous over-expression of the key components of photosynthetic sucrose biosynthesis, cytosolic fructose-1,6-bisphosphatase (cFBPase), and TPT in *Arabidopsis* resulted in enhanced growth and increased fresh weights than wild-type (WT) plants, indicating its role in growth enhancement (Cho et al. 2012), which may contribute to the vigor noticed in hybrids as compared to their parents.

The transcript F 22 (F₁nBP transcript pattern) showed 95% similarity to S-adenosylmethionine decarboxylase 3 (SAMDC3) (Sobic.006G135600.4), a rate-limiting gene associated with polyamine biosynthesis pathway that plays critical roles in plant growth and development (Basu et al. 2014; Mo et al. 2016) and extensively controls the transcriptional and post-transcriptional levels, thereby helping plants respond to abiotic stresses (Hu et al. 2005). The F 25 (F₁nBP transcript pattern) transcript exhibited 99% similarity to *Sorghum bicolor* putative 4-hydroxy-4-methyl-2-oxoglutarate aldolase 2 (Sobic.003G069500.1) that helps in the regulation of RNA metabolic processes. The differentially expressed transcript F 28 (F₁nBP transcript pattern) showed a 99% similarity to *Sorghum bicolor* DEAD-box ATP-dependent RNA helicase 14 (Sobic.003G050700.2). DEAD-box proteins perform diverse functions in basic cellular processes (DNA replication, DNA repair, ribosome biogenesis, and transcription), quality control mechanisms associated with gene expression, nuclear export of mRNA, mRNA splicing, and others, thereby playing an important role in the regulation of plant growth and development (Gong et al. 2005; Linder 2006; Jankowsky and Fairman 2007; Xu et al. 2015). The predicted product of the transcript F 36 (F₁nBP expression pattern) exhibited a 99% similarity to *Sorghum bicolor* probable cellulose synthase A catalytic subunit 3 [UDP-forming] (Sobic.002G118700.1). This gene is involved in the cellulose biosynthesis pathway in plants, which is a part of Glycan metabolism (Peng et al. 2002; Read and Bacic 2002). Some of the differentially expressed transcripts identified in this study are associated with important biochemical pathways such as glycolysis, pyrimidine, sucrose, polyamine, and cellulose biosynthesis. Data from transcriptomic studies in recent years have emphasized the role of certain key biochemical pathways that may be ideal for the manifestation of heterosis in crop plants (Baranwal et al. 2012). The transcript F 18 (UET pattern) exhibited a 98% similarity to OSIGBa0126B18.9 protein (Sobic.006G214400.1). Some of the transcripts (F 10, F 20, F 30, F 34, F 45, and F 49) exhibited similarities with *Sorghum bicolor* uncharacterized proteins, of which, F 20, F 34, and F 49 had F₁nBP patterns and F 10, F 30, and F 45 exhibited UPnF₁, BPnF₁, and UPF₁ transcript patterns, respectively.

Table 5 Details of differentially expressed transcripts eluted from DD-PAGE gels and BLASTX results

Fragment code	Expression pattern	Size (bp)	Eluted from	Growth stage	GenBank ID	Putative gene	Homology %	Accession no. of putative gene
F5	F ₁ nBP	244	27A×C 43	Booting	MG436951	<i>Sorghum bicolor</i> UMP-CMP kinase 4	99	Sobic.007G223100.1
F10	UPnF ₁	205	CB 29	Flowering	MG436948	<i>Sorghum bicolor</i> uncharacterized LOC8063969	98	Sobic.006G214400.1
F12	BPnF ₁	355	27B	Booting	MG436945	<i>Sorghum bicolor</i> two-component response regulator-like PRR73	97	Sobic.001G411400.1
F16	F ₁ nBP	430	7A×C 43	Flowering	MG436937	Triose phosphate/ phosphate translocator, chloroplastic	99	Sobic.003G002300.1
F18	UET	327	RS 627	Flowering	MG436949	Similar to OSIG-Ba0126B18.9 protein	98	Sobic.006G214400.1
F20	UET	593	27A×RS 673	Booting	MG436938	<i>Sorghum bicolor</i> uncharacterized protein	99	Sobic.006G214400.1
F22	F ₁ nBP	217	27A×RS 673	Booting	MG436947	S-adenosyl-methionine decarboxylase 3 (SAMDC3)	95	Sobic.006G135600.4
F25	F ₁ nBP	405	7A×C 43	Booting	MG436939	<i>Sorghum bicolor</i> putative 4-hydroxy-4-methyl-2-oxoglutarate aldolase 2	99	Sobic.003G069500.1
F28	F ₁ nBP	334	7A×RS 673	Vegetative	MG436943	<i>Sorghum bicolor</i> DEAD-box ATP-dependent RNA helicase 14	99	Sobic.003G050700.2
F30	BPnF ₁	205	C 43	Booting	MG436942	<i>Sorghum bicolor</i> uncharacterized protein	99	Sobic.006G214400.1
F34	F ₁ nBP	244	7A×CB 26	Booting	MG436952	<i>Sorghum bicolor</i> uncharacterized protein	99	Sobic.003G170900.2
F36	F ₁ nBP	421	7A×CB 26	Flowering	MG436940	<i>Sorghum bicolor</i> probable cellulose synthase A catalytic subunit 3 [UDP-forming]	99	Sobic.002G118700.1
F42	F ₁ nBP	407	27A×RS 673	Vegetative	MG436950	NADP-dependent glyceraldehyde-3-phosphate dehydrogenase (LOC8080604)	98	Sobic.007G140700.1
F45	UPF ₁	205	7A×RS 673	Booting	MG436941	<i>Sorghum bicolor</i> uncharacterized protein	98	Sobic.006G214400.1

Table 5 (continued)

Fragment code	Expression pattern	Size (bp)	Eluted from	Growth stage	GenBank ID	Putative gene	Homology %	Accession no. of putative gene
F46	UET	327	7A×RS 673	Vegetative	MG436946	<i>Sorghum bicolor</i> putative disease resistance protein RGA3	99	Sobic.002G169633.1
F49	F ₁ nBP	326	7A×CB 26	Booting	MG436944	<i>Sorghum bicolor</i> uncharacterized protein	98	Sobic.004G010500.1

UET—universally expressed transcript (parents and F₁ hybrid); F₁nBP—transcript expressed only in the hybrid, but not in both the parents; UPF₁—transcript expressed in any one of the parents and F₁ hybrid; BPnF₁—transcript expressed in both the parents, but not in F₁ hybrid, and UPnF₁—transcript expressed in any one of the parents, but not in the other parent and F₁ hybrid

Correlation between differential gene-expression patterns and agronomic traits

Correlation analysis between differential transcript patterns detected in F₁ hybrids and their corresponding parents at

critical developmental stages and heterosis for grain yield and its component traits in this study revealed both positive and negative correlations with few patterns (Table 6). A negatively correlation between mid-parent heterosis for days to flowering (-0.49 at $p < 0.05$) and primary branches/

Table 6 Correlation between differential gene-expression patterns at critical developmental stages and heterosis for grain yield and its component traits pooled over 2 years

Trait	Growth stage	F ₁ nBP		UPF ₁		BPnF ₁		UPnF ₁	
		MPH	BPH	MPH	BPH	MPH	BPH	MPH	BPH
Days to 50% flowering	Vegetative	-0.49*	-0.12	-0.03	-0.26	-0.52*	-0.22	0.12	0.00
	Booting	0.13	-0.04	0.36	0.22	0.08	-0.07	-0.07	0.11
	Flowering	-0.40	-0.17	0.47*	0.39	0.35	0.02	-0.07	0.06
Plant height	Vegetative	0.07	0.02	0.26	0.15	0.06	-0.12	-0.41	-0.36
	Booting	0.02	0.22	0.29	0.39	-0.37	-0.20	0.16	0.04
	Flowering	0.06	-0.10	-0.01	0.11	0.23	0.30	-0.19	-0.29
Panicle length	Vegetative	-0.17	-0.07	0.12	-0.08	0.04	0.10	-0.11	-0.02
	Booting	0.04	0.22	0.36	0.04	-0.20	-0.06	0.13	-0.03
	Flowering	0.03	0.10	-0.01	-0.02	0.33	0.07	-0.41	-0.19
Panicle weight	Vegetative	-0.32	-0.24	-0.26	-0.20	0.17	0.17	0.05	0.01
	Booting	0.33	0.24	0.37	0.31	-0.01	-0.05	0.09	0.10
	Flowering	0.11	0.10	-0.01	-0.01	0.23	0.20	-0.53*	-0.44*
Primary branches/panicle	Vegetative	-0.46*	-0.50*	0.20	0.29	-0.10	-0.27	0.16	0.15
	Booting	0.33	0.27	0.35	0.41	0.10	0.04	-0.16	-0.10
	Flowering	-0.10	-0.17	-0.08	-0.01	0.63**	0.61**	-0.73**	-0.63**
Grain yield/plant	Vegetative	-0.29	-0.20	-0.30	-0.24	0.18	0.18	0.02	-0.01
	Booting	0.33	0.24	0.35	0.24	-0.09	-0.09	0.13	0.09
	Flowering	0.12	0.12	0.05	0.04	0.22	0.15	-0.51*	-0.37
Test weight	Vegetative	-0.02	-0.11	-0.04	-0.05	0.64**	0.52*	-0.14	-0.02
	Booting	-0.16	-0.21	0.09	-0.01	-0.29	-0.37	0.27	0.27
	Flowering	0.39	0.43	-0.27	-0.16	0.09	-0.02	-0.31	-0.12

The values represented in * and ** are significant at $p < 0.05$ and at $p < 0.01$, respectively

Pooled over 2012 and 2013

F₁nBP—transcript expressed only in the hybrid, but not in both the parents; UPF₁—transcript expressed in any one of the parents and F₁ hybrid; BPnF₁—transcript expressed in both the parents, but not in F₁ hybrid; and UPnF₁—transcript expressed in any one of the parents, but not in the other parent and F₁ hybrid

MPH mid-parent heterosis, BPH better-parent heterosis

panicle (-0.46 at $p < 0.05$) and F_1 BP transcript pattern was observed at the vegetative stage. This observation was similar to that reported by Xiong et al. (1998) and Wang et al. (2007), but contrary to observations made by Sun et al. (2004), who noticed a positive correlation of the transcripts detected only in F_1 hybrids but not in either of the parents with heterosis. The UPn F_1 -transcript pattern at the flowering stage was negatively correlated with mid-parent heterosis as well as better-parent heterosis for panicle weight and primary branches/panicle at flowering stage, which implies that the repression of genes in hybrids that were expressed in either male or female parents is not favourable for the expression heterosis. These results are in agreement with the report of Sun et al. (2004) in which the negative correlation between the differentially expressed fragments occurring in only one of the two parents or in F_1 hybrids and heterosis was demonstrated. On the contrary, Wang et al. (2007) observed a positive correlation between mid-parent heterosis and UNP-transcript pattern, which is similar to UP F_1 transcript pattern and mid-parent heterosis for days to flowering in this study. A significant negative correlation was observed between mid-parent heterosis value of days to flowering with BPn F_1 patterns (-0.52 at $p < 0.05$) at vegetative stage. The presence of positive correlation between BPn F_1 transcript pattern and the mid-parent heterosis and heterobeltiosis for test weight (0.64 at $p < 0.01$ and 0.52 at $p < 0.05$) and primary branches/panicle (0.63 and 0.61 at $p < 0.01$) during vegetative and flowering stages, respectively, implies that genes silenced in hybrids may contribute to the expression of heterosis. Similarly, Wu et al. (2001) also reported that the genes expressed in parents but restrained in hybrids may be responsible for heterosis in maize. The correlation analysis between differential gene-expression patterns with mid-parent heterosis and better-parent heterosis is given in Table 6. Even though some studies reported a lack of correlation between differential gene-expression patterns and agronomic performance of hybrid in rice and wheat (Xiong et al. 1998, 1999; Wu et al. 2001), the present study detected some correlations between differential transcript patterns and heterosis for grain yield and its components.

Conclusion

In conclusion, the present investigation analyzed the transcript expression patterns in a set of sorghum hybrids and their parental lines at three critical stages of development through differential display analysis resulting in the identification of differentially expressed transcripts (60.39%) and universally expressed transcripts (39.61%). Sequence analysis of 16 transcripts revealed that they code for genes such as NADP-dependent glyceraldehydes-3-phosphate dehydrogenase, UMP-CMP Kinase 4, two-component response

regulator-like PRR73, triose-phosphate translocator, S-adenosylmethionine decarboxylase 3, 4-hydroxy-4-methyl-2-oxoglutarate aldolase 2, DEAD-box ATP-dependent RNA helicase 14 and cellulose synthase A catalytic subunit 3, which are involved in basic cellular processes, ATP production, cellulose biosynthesis, assimilate partitioning during photosynthesis, polyamine biosynthesis, pyrimidine biosynthesis, and regulation of plant growth and development. Analysis of transcript patterns between high and low heterotic hybrids revealed involvement of over-dominance and dominance expressions in the manifestation of heterosis. Significant positive as well as negative correlations were observed between differential expression patterns with heterosis for grain yield and its components revealing the complex nature of the biological phenomenon of heterosis. Even though the putative function of a limited number of differentially expressed transcripts related to heterosis were determined through sequence analysis, this study provided vital information on the nature of genes involved in heterotic expression, offering scope for the identification of more such genes, which will help in the deciphering the network of genes associated with grain yield heterosis in sorghum.

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Compliance with ethical standards

Conflict of interest We disclose that we do not have any actual or potential conflict of interests including any financial, personal or other relationships with other people or organizations.

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