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Studies on involvement of *Wrinkled1* transcription factor in the development of extra-long staple in cotton

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Abstract

In the present investigation, fiber transcriptome of *Gossypium hirsutum* producing short-fiber (JKC703, JKC737, JKC783) and long-fiber (JKC725, JKC777, Guazuncho 2) was compared with the transcriptome of extra-long fiber producing *Gossypium barbadense* (VH8-4602). By meta-analysis, we identified 1431 differentially expressed genes among the genotypes of different fibre lengths. Of these, 574 genes were up-regulated while 844 were down regulated in *G. barbadense* as compared to *G. hirsutum.* Expression of 5 genes related to fiber development was tested in local germplasm of *G. barbadense* (Bar14/5) and used *G. hirsutum* (MNH 886) using real-time RT-PCR. *G. arboreum* (FDH786) was as a sample with unknown expression. Expression pattern of an ethylene responsive transcription factor *wrinkeled-1 (wri1*), a vacuolar processing enzyme (*vpe*) and eceriferum3 (*cer3*) gene showed close association with fiber lengths. Moreover, cotton *wri1* expressed in apical-meristem and bolls and showed significantly higher expression in fiber of *G. barbadense* as compared to *G. hirsutum* and *G. arboreum* during development. Level of Transcription of *wri1* was enhanced in fiber during ball development while it remained unchanged in fiber-less seeds.

Key words: Cotton-fiber, elongation, transcriptomics, microarray

Introduction

Cotton-fiber is an important commodity and most frequently used natural fiber of the world (Stiff and Haigler 2012). There are four commercially important species of *Gossypium*. Tetraploid species are *Gossypium hirsutum* and *G. barbadense* while diploid species include *G. arboreum* and *G. herbaceum*. *G. barbadense* produces very long, fine and strong fibers (Avci et al. 2013) which are easier to spin and are preferred in textile industry as compared to *G. hirsutum*. Despite having the characteristics of elite fiber, *G. barbadense* is cultivated only on a small area owing to its low yield and limited adaptability.

Scientists have identified genes regulating osmo-regulation (Wang et al. 2010b), callose-pluging of plasmodesmata (Ruan 2007), cytoskeleton modifications (Cosgrove et al. 2002) and starch biosynthesis (Taliercio 2011) during fiber development. Role and interaction of many phytohormones i.e. abscisic acid (Dasani and Thaker 2006), gibberellic acid (Aleman et al. 2003), brassinosteroids (Sun et al. 2005) and ethylene-biosynthesis (Shi et al. 2006) during fiber development has also been highlighted.

Many scientists have utilized global transcriptome analysis to identify the genes involved in fiber development, and have reported a number of differentially expressed genes (DEG). However, only few genes have so far been characterized while the role of a large number of DEGs remains un-explored. In that study, we have used public microarray raw data produced in independent cotton fiber developmental studies and performed meta-analysis to identify the genetic factors which showed differential expression consistantly.

Materials and methods

Meta-analysis

For meta-analysis, gene expression omnibus (GEO)

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portal on National Center for Biotechnology Information (NCBI) was explored to find all the data available on cotton-fiber transcriptomics. For minimizing the variability among experiments, we selected experiments containing 12DPA fiber-transcriptomics data produced using affymetrix platforms (GPL8672). Raw data files (as given in Table 1) were downloaded and analyzed using Genespring GX (Agilent) bioinformatics tools. The whole fiber transcriptome of Extra-long fiber (VH8-4602), long-fiber (JKC725, JKC777, Guazuncho 2) and short-fiber (JKC703, JKC737, JKC783) were compared (Table 1). The genes which were significantly differentially expressed among these genomes at the p-value <0.01 were identified. Functional categorization of genes was performed using Gene Ontology analysis.

Plant growth and sampling

Three local genotypes of cotton i.e., *G. hirsutum* L. var. MNH 886, *G. barbadense* L. var. Bar 14/5 and *G. arboreum* var. FDH 786 (Desi cotton) were sown in the fields of University of the Punjab, Lahore, Pakistan during cotton growing season. On the onset of flowering, leaf, meristem, bract, petal and ovary samples were collected. On the day of flower opening/ anthesis, flowers were tagged. The day of the opening of flower was taken as 0DPA. Cotton-bolls at different developmental stages were collected, dissected, locules were snap-frozen in liquid nitrogen and stored at -80°C. Each sample represented at least 3 biological replicates.

Primer design and polymerase chain reaction (PCR)

Gene-specific primers (20-22mer) were designed using primer3 online primer designing tool. The sequences of primers used in this study are given in Supplementary Table S1 (available online at http:// www.isgpb.co.in). We used 0.2mM of dNTPs, 1.5mM $MgCl_2$, 0.3 μM of each forward and reverse primer, 1X Taq DNA-Polymerase buffer (100nM Tris-HCl-pH8.8, 500mM KCl, 0.8% Tween 20) for amplification of PCR-products. The optimized thermocycling coditions were intial-denaturation at 95^{o}C for 3 minutes then 40 cycles of denaturation at 95^{o}C, annealing at 60^{o}C and extension at 72^{o}C (each for 30sec) followed by final-extension at 72^{o}C for 10min. The amplificaiton mixture was electrophoresed on 2% agarose gel containing 0.5μg/mL of ethidium bromide and 0.5X Tris-Acetate-EDTA buffer at 100 volts for 40min.

Complementary-DNA (cDNA) library construction and real-time reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was extracted from fibers at 3DPA, 5DPA, 7DPA, 14DPA and 17DPA by using hot-borate method (Wan and Wilkins 1994). cDNA was constructed from 5 μg of DNase-free RNA by using SuperScript^{TM}II RT First Strand cDNA Synthesis kit (Invitrogen). Real-time RT-PCR was performed using Maxima 2X SYBR green master mix (Thermoscientific) in IQ5 real-time thermocycler (Biorad). cDNA quantities were normalized using EF1 and Tub genes as internal controls. Normalized cycle threshold (CT) values were used to calculate relative-expression and standard-deviation using ΔΔCT method. Analysis of variance (ANOVA) and Bonferroni post test was performed to find the significance of variation among different samples.

Cotton-fiber analysis

Mature cotton-fibers were handpicked from plants of experimental varieties and were sent to National Institute of Biotechnology and Genetic Engineering (NIBGE, Faisalabad) for fiber quality analysis. High Volume Instrument (USTER HVI 1000) was used to determine the fiber properties using standard protocol (Uster 1999; Asif et al. 2008).

Results and discussion

Transcriptional dynamics during cotton-fiber elongation.

In this study, meta-analysis was done for 24,132 cotton genes in 19 affymetrix data sets (Table 1), and found that 1,431 genes showed differential expression among different species (p-value <0.01). Of those, 574 genes were upregulated while 844 genes were down-regulated in *G. barbadense* as compared to *G. hirsutum*. Further, 5 genes showed positive while 23 genes showed negative correlation with fiber-elongation in *G. hirsutum* as against *G. barbadense* (Fig. 1a). Expression profile of short and long fibers of *G. hirsutum* didn't show much variation but in very-long fibers (*G. barbadense*) gene expression was quite divergent from the other two (Fig. 1b).

Functional categorization of differentially expressed genes was performed using Gene Ontology analysis to identify the over represented functional classes. O-acyltransferase activity, flavonoid biosynthetic process, lignin biosynthesis, kinase activity genes, auxin homeostasis and hydrogen

Table 1. Data sets used in meta-analysis

Geo accession	No. of number	Sample accession samples	Organism	Genotype	Fiber length*
GSE43528	4	GSM1064952	*Gossypium barbadense*	VH8-4602	Extra-long/ very-long fiber
		GSM1064951	*Gossypium barbadense*	VH8-4602	Extra-long/ very-long fiber
GSE36228	15	GSM1064954, GSM1064953, GSM884241, GSM884239, GSM884240, GSM884275, GSM884276, GSM884277, GSM884257,	*Gossypium hirsutum*	Guazuncho 2 JKC725, JKC8777	Long-fiber
		GSM884258, GSM884259, GSM884293, GSM884294, GSM884295, GSM884310, GSM884311, GSM884312		JKC703, JKC783& JKC737	Short-fiber

*Extra-long fiber length (>26mm), long fiber (24.5-26mm) and short fiber (21.5-23.5) as described by Nigam et al. 2014.

peroxide response related categories were over-represented (Supplementary Table S2, available online at http://www.isgpb.co.in).

Expression of selected genes in local germplasm

Out of all differentially expressed genes, five genes *viz*., (*wri1*, *cer3*, hsl1, e3 and *vpe*) were selected on the basis of their functional relevance in fiber development for validation in local germplasm of *G. barbadense*, *G. hirsutum* and *G. arboreum* using real-time RT-PCR. The validation was at 7DPA.

Expression of all tested genes showed higher expression in *G. barbadense* as compared to *G. hirsutum* (Fig. 2c). *wri1* transcript accumulation in *G. barbadense* was 3.5 fold higher than *G. hirsutum* and 18.7 fold higher than *G. arboreum* which correlate with fiber lengths of these germplasms (Table 2). Vacuolar processing enzyme (*vpe*) transcript accumulation also correlate with fiber length i.e. highest expression in *G. barbadense* (10 fold) followed by 7 fold in *G. hirsutum* as compared to *G. arboreum*. Transcript accumulation of *cer3* gene was 2.7 fold higher in *G. barbadense* and 2.1 fold higher in *G. hirsutum* in comparison with *G. arboreum*. However, the increase in the expression of the other two genes *viz*., e3 and *hsl1* was not significant.

Fiber quality assessment of different cotton species

Fiber properties i.e. micronaire, short-fiber percentage, upper half mean length (fiber length), strength and elongation percentage were determined in fibers of the three genotypes under study. Micronaire value of fiber of *G. barbadense* (3.81) and *G. hirsutum* (3.84) was significantly lower as compared to *G. arboreum* 6.15 (Table 2) which indicated that fibers are finer in the above two varieties. Short-fiber percentage (weight of the fiber <12.7 mm) was 12.4 % in *G. barbadense*, 13.8 % in *G. hirsutum* and had a marked difference from 20.6 % in *G. arboreum* (Table 2). *G. barbadense* produced the longest fibers of 27.05 mm, *G. hirsutum* 24.54 mm and *G. arboreum* 20.60 mm (Table 2). The data revealed the highest strength value i.e., 27.10 g/tex for *G. barbadense* among all other varieties under study. Fiber elongation (stretching of the fiber at breaking load) in *G. barbadense* exhibited the highest percentage of 6.5 in comparison with 5.76 and 5.76 in *G. arboreum* and *G. hirsutum*, respectively (Table 2). All the data indicated that *G. barbadense* and *G. hirsutum* possesses fiber trait that are required for spinning and processing of cotton-fiber in the textile industry.

Expression of Wrinkeled-1 during cotton-fiber development

Fiber quality assessment data and gene expression data indicated that *cer3*, *wri1* and *vpe* genes are expressed at higher level in genotypes producing longer fiber (Fig. 2). Since, *wri1* showed highest differences in expression among genotypes so we selected it for further studies.

We have analyzed the *wri1*-expression in the longest fiber producing species (*G. barbadense*) and shortest fiber producing species (*G. arboreum*) at important stages during fiber development. In cotton, 3 DPA marks the initiation stage, 5 DPA marks start of elongation while 7 DPA marks rapid elongation. At 14 DPA elongation slows down and 17 DPA marks the transition from elongation to cellulose deposition stage

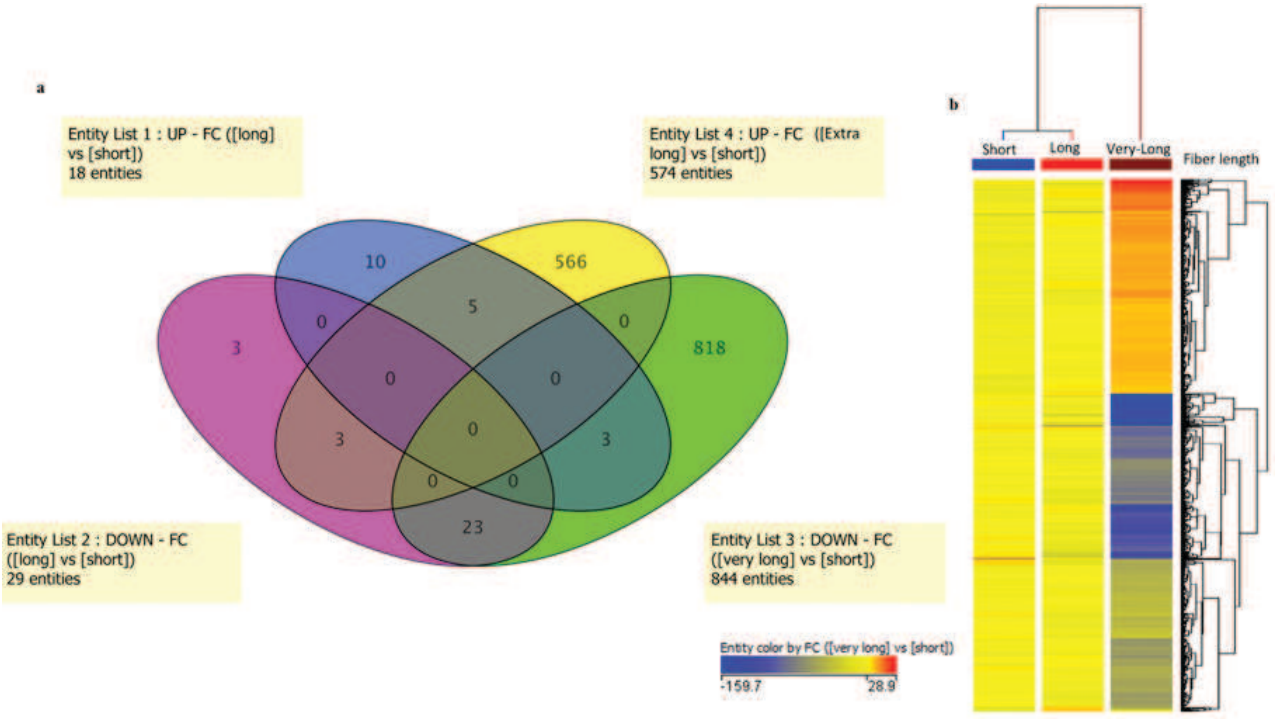

Fig. 1. Expression profile of differentially expressed genes in short (*G. hirsutum*), long (*G. hirsutum*) and very long (*G. barbadense*) fibers. (a) Venn diagram showing overlapping of upregulated and downregulated genes. (b) Heat map showing the expression levels (Fold change) of genes in different samples

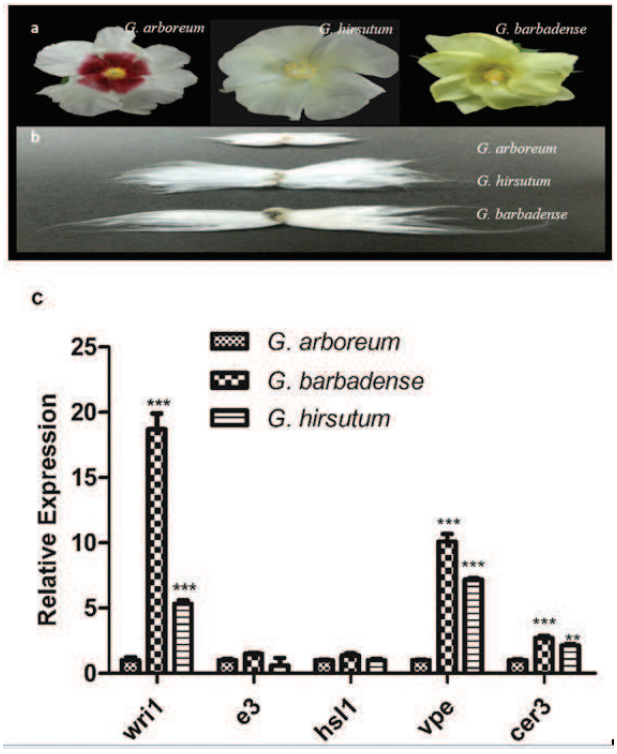

Fig. 2. Comparison in genotypes of cotton. (a) Flower morphology (b) Fiber-length (c) Transcriptional analysis of fiber related genes (*wri1*, *cer3*, *hsl1*, *e3* and *vpe*). * indicate p-value ≤0.01, ** indicate p-value ≤0.05 and NS indicate p-value >0.05**

We found that the expression of *wri1* was significantly higher in extra-long staple producing cotton genotype (*G. barbadense*) as compared to short-fiber producing genotype (*G. arboreum*) during the

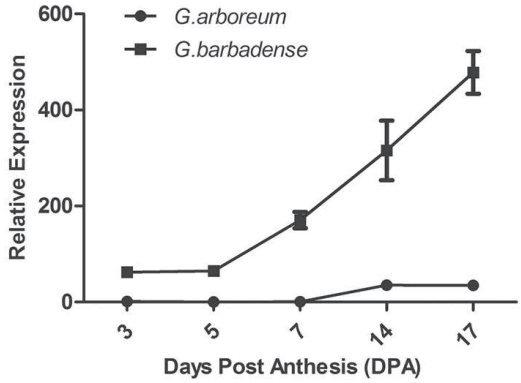

Fig. 3. *wri1* expression-dynamics during fiber-development in *G. barbadense* and *G. arboreum*

development of fiber (Fig. 3). Expression of *wri1* which is an ethylene responsive transcription factor is highest during the transition of fiber-elongation stage to secondary cell wall synthesis stage (17 DPA) in both the genotypes.

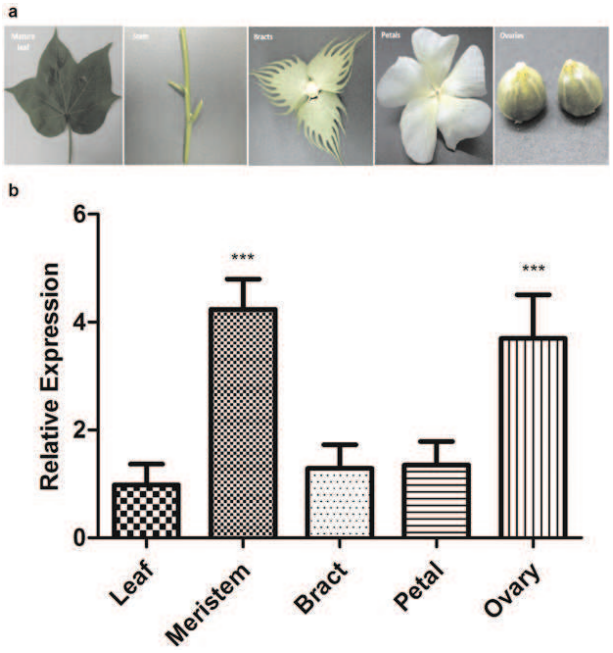

Fig. 4. *wri1*-expression in different tissues of *G. barbadense* (a) Tissues used for expression analysis. (b) Expression of *wri1* gene in different tissues. ***indicate p-value ≤0.01

expression in cotton seed. We separated lint fibers from seeds at different developmental stages and studied *wri1*-expression in seeds and fibers separately. Results showed that the expression of this gene is higher in seeds at early stages of embryo development (5 and 7 DPA) while after 7 DPA transcript

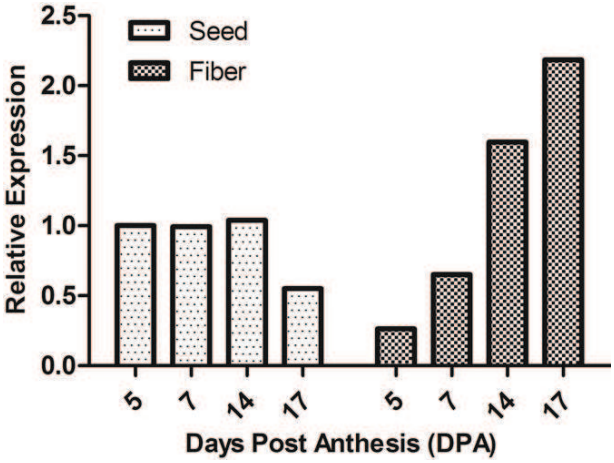

Fig. 5. *wri1*-expression during seed and fiber development in *G. barbadense*

Table 2. Fiber characteristics of three *Gossypium* species using HVI system

Characteristics	*G. arboreum* L. var. FDH 786	*G. hirsutum* L. var. MNH886	*G. barbadense* L. var. Bar 14/25	Probability value
Micronaire	6.15±0.07	3.84±0.03	3.81±0.01	<0.0001
Length (mm)	20.76±0.56	24.54±0.18	27.05±0.15	<0.0001
Short fiber (%)	20.60±1.17	13.80±0.81	12.40±0.34	<0.0001
Strength (gm/tex)	19.97±0.47	21.77±0.20	27.10±0.62	<0.0001
Elongation (%)	5.76±0.37	5.06±0.05	6.50±0.264	<0.01

Wrinkeled-1 transcript accumulation in different tissues of cotton

In order to investigate whether *wri1* is expressed in all tissues of the plant or its expression is fiber-specific, we analyzed the transcription of *wri1* in different tissues of the cotton plant. As shown in Fig. 4b, *wri1* has significantly higher expression in ovary (3.7 fold) and apical meristem (4.2 fold) as compared to other vegetative tissues, which indicates its role in cotton boll development.

Wrinkeled-1 gene is previously described to be involved in embryo-development (Baud et al. 2009) and fatty acid biosynthesis in *Arabidopsis thaliana* (Focks and Benning 1998, Maeo et al. 2009) and oil palm (Ma et al. 2013). So it was important to study its accumulation is significantly higher in 14 and 17 DPA fiber as compared to seeds (Fig. 5). Fibers at this stage undergo transition from elongation to cellulose deposition. These results indicated that *wri1* might play a dual role in cotton i.e., promoting embryo development and fiber development.

The homologues of *wri1* are ethylene-responsive transcription factors (Riechmann et al. 2000). As ethylene plays a role in transition from primary cell wall to secondary cell wall biosynthesis so *wri1*-transcription could be involved in triggering the expression of genes involved in secondary cell wall biosynthesis. Thus identification of *wri1* targets and studying their role could be important in establishing the role of *wri1* in this transition.

Authors' contribution

Conceptualization of research (UQ, FA); Designing of the experiments (UQ); Contribution of experimental materials (FA, MA); Execution of field/lab experiments and data collection (FA, UQ); Analysis of data and interpretation (SY); Preparation of manuscript (UQ).

Declaration

The authors declare no conflict of interest.

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Supplementary Table S1. Gene-specific Primers for RT-PCR

No.	Primer Name	Genbank Accession	Primer Sequence 5'-3'
1	Tub F	XM_012628653	TGAGGAAAGAAGCCGAAAAC
2	Tub R		GGAAGGGAACACAGAGAAAGTG
3	EF1aF	DQ174257	TTCAGTGGTCAATCCAGAAGG
4	EF1aR		GGATGTCTACAAGATCGGTGGT
5	VPE F	CO100487	GCATTTCCAGTCATCAACAAGA
6	VPE R		GAAGCCATGTCCCACAGAA
7	Cer3 F	XM_012587010	GTGATAAAGATGATGTGTGGGAGT
8	Cer3 R		CCTTGTTGAAGTGATGAAACCA
9	E3 F	DR458048	CACCAGTTTACATGGCCTTTCT
10	E3 R		TCAGTTCCTGTCTATCACCACCT
11	Wri1 F	DW505003.1	GCCGGTATTGAGCCTACTGA
12	Wri1 R		TCATCATCCATCACGCATTT
13	HSL F	CO126595	ACTGCGTGTGAACGAGAAGAG
14	HSL R		ATCGAGCTTGGAATCGAGAA

Supplementary Table 2 (S2). Functional classification of differentially expressed genes in extra-long fiber as compared to shorter fiber

GO ACCESSION	GO Term	p-value
GO:0008374	O-acyltransferase activity	4.37E-05
GO:0009809	Lignin biosynthetic process	1.71E-04
GO:0004674|GO:0004695	Protein serine/threonine kinase activity	2.55E-04
GO:0004672|GO:0050222	Protein kinase activity	3.80E-04
GO:0009808	Lignin metabolic process	4.17E-04
GO:0006468	Protein phosphorylation	4.59E-04
GO:0016301	Kinase activity	6.56E-04
GO:0016773	Phosphotransferase activity, alcohol group as acceptor	6.56E-04
GO:0016310	Phosphorylation	7.76E-04
GO:0016772	Transferase activity, transferring phosphorus-containing groups	7.76E-04
GO:0016740	Transferase activity	0.001
GO:0010167	Response to nitrate	0.001
GO:0009962	Regulation of flavonoid biosynthetic process	0.001
GO:0047205	Quinate O-hydroxycinnamoyltransferase activity	0.001
GO:0009934	Regulation of meristem structural organization	0.001
GO:0047172	Shikimate O-hydroxycinnamoyltransferase activity	0.001
GO:0010480	Microsporocyte differentiation	0.001
GO:0009963	Positive regulation of flavonoid biosynthetic process	0.001
GO:0016411	Acylglycerol O-acyltransferase activity	0.001
GO:0050734	Hydroxycinnamoyltransferase activity	0.001
GO:0008353	RNA polymerase II carboxy-terminal domain kinase activity	0.001
GO:0050737	O-hydroxycinnamoyltransferase activity	0.001

GO:0004622|GO:0045126	Lysophospholipase activity	0.001
GO:0000502	Proteasome complex	0.001
GO:0003993	Acid phosphatase activity	0.001
GO:0003846	2-acylglycerol O-acyltransferase activity	0.001
GO:0016747	Transferase activity, transferring acyl groups other than amino-acyl groups	0.001
GO:0009699	Phenylpropanoid biosynthetic process	0.001
GO:0044550	Secondary metabolite biosynthetic process	0.001
GO:0030554	Adenyl nucleotide binding	0.001
GO:0005524	ATP binding	0.002
GO:0032559	Adenylribonucleotide binding	0.002
GO:0016746|GO:0008415	Transferase activity, transferring acyl groups	0.002
GO:0009698	Phenylpropanoid metabolic process	0.003
GO:0001882	Nucleoside binding	0.003
GO:0017076	Purine nucleotide binding	0.003
GO:0032550	Purine ribonucleoside binding	0.003
GO:0035639	Purine ribonucleoside triphosphate binding	0.003
GO:0001883	Purine nucleoside binding	0.003
GO:0032549	Ribonucleoside binding	0.003
GO:0032555	Purine ribonucleotide binding	0.003
GO:0010252	Auxin homeostasis	0.003
GO:0042542	Response to hydrogen peroxide	0.004
GO:0048653	Anther development	0.004
GO:0004620	Phospholipase activity	0.004
GO:0009749	Response to glucose	0.004
GO:0097472	Cyclin-dependent protein kinase activity	0.004
GO:0004693|GO:0016537	Cyclin-dependent protein serine/threonine kinase activity	0.004
GO:0010043	Response to zinc ion	0.004
GO:0016298	Lipase activity	0.004
GO:0071944	Cell periphery	0.004
GO:0032553	Ribonucleotide binding	0.004
GO:0097367	Carbohydrate derivative binding	0.004
GO:0006796	Phosphate-containing compound metabolic process	0.004
GO:0006793	Phosphorus metabolic process	0.005
GO:0005886|GO:0005904	Plasma membrane	0.006
GO:0000166	Nucleotide binding	0.007
GO:1901265	Nucleoside phosphate binding	0.007
GO:0010075	Regulation of meristem growth	0.007
GO:0048509	Regulation of meristem development	0.007
GO:0048610	Cellular process involved in reproduction	0.007
GO:0033612	Receptor serine/threonine kinase binding	0.007
GO:0005730	Nucleolus	0.007
GO:0005102	Receptor binding	0.007
GO:0000302	Response to reactive oxygen species	0.007
GO:0046777	Protein auto-phosphorylation	0.007