RNA Seq Analysis Reveal Differential Expression of Genes Associated With Metabolic Reprogramming, Defense and Signaling in Two Contrasting Indigenous Aromatic Rice Cultivars Differing in Redox Regulatory Properties Under Drought Stress

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RNA seq analysis reveal differential expression of genes associated with metabolic reprogramming, defense and signaling in two contrasting indigenous aromatic rice cultivars differing in redox regulatory properties under drought stress

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Abstract

Background

Though there are at present seventy indigenous aromatic rice cultivars being cultivated in West Bengal, India, the information regarding their genetic regulation of drought tolerance are scanty. Previous work from this laboratory has screened indigenous aromatic rice cultivars Badshabhog and Jamainadu as drought tolerant redox competent and drought susceptible redox incompetent germplasms respectivelybased on redox metabolomic investigation. In this backdrop, the present work comprehended to compare two indigenous aromatic rice cultivars for unfolding genetic regulation of drought stress.

Results

The result of comparative transcriptomic study through RNA-seq analysis in general revealed significant cultivar specific expression of genes under post-imbibitional dehydration stress as well as genes that are expressed in both the experimental cultivars (*Oryza sativa* L., cultivars Badshabhog and Jamainadu) with differential transcript abundance. 3482 and 3866 genes were found to be significantly down-regulated and up-regulated respectively when we compared between post-imbibitional dehydration stress-raised indigenous aromatic rice cultivars Badshabhog and Jamainadu. Transcripts selected for heatmap generation with proper annotation revealed 50 genes that are significantly expressed genes which are mainly involved in redox functions, signaling, membrane trafficking, replication and protein synthesis etc. On the contrary, genes which got highly expressed in Badshabhog found to be mainly concerned with carbondioxide concentrating mechanism (NADH-dependent malic enzyme), peroxysomal biogenesis, protein modification, protein synthesis, mitochondrial electron transport chain functioning, inercellular protein transport, histone demethylation

associated with developmental process, regulation of apoptosis etc. Further Badshabhog exhibited greater GO (gene ontology) distribution so far as signaling, antioxidant functioning, biological and metabolic regulation, transcriptional and translational regulation etc. with significantly higher percentage of antioxidant genes. KEGG (kyoto encyclopedia of genes and genomes) pathway analysis showed several signal transduction pathways (abscisic acid, brassinosteroid, salicylic acid, jasmonic acid and ethylene) being influenced by drought stress.

Conclusion

The overall result of comparative transcriptomic investigation suggests that indigenous aromatic rice cultivars Badshabhog and Jamainadu explored varying strategies to deal with post-imbibitional dehydration stress. The cultivar Badshabhog exhibited a significantly greater molecular reprogramming presumably more through up-regulation of metabolic and energy demanding processes along with implementing better signaling strategies, antioxidative defense mechanism, DNA repair and transcriptional regulation *vis-a-vis* the cultivar Jamainadu.

Key words: Aromatic Rice, Drought tolerance, Redox regulation, RNA-seq, Transcriptome.

Background

Last few decades witnessed significant demand of aromatic rice internationally, due to their kernel quality and aroma [1]. Since the production of aromatic rice is largely threatened by environmental challenges, several comities across the globe put their effort for improving the existing aromatic rice cultivars based on genotype-environment interaction studies [2]. The overall observation revealed inverse relationship between yield and aroma quality, productivity under stress [3]. So, the target of achieving superior genotypes through selection

of phenotypes of multiple complex traits is difficult due to varied genotype-environment interaction, genetic drag and inferior heritability [4]. Therefore, present time demands improvement of aromatic rice through the comprehensive study covering well annotated accession wise transcriptomic investigation under fluctuating environmental conditions *vis-a-vis* their standard metabolic and physiological phenotypes. So, comprehensive study exhibiting complexity of transcriptional regulation in diverse genotypes under prominent stress condition like drought is essential to augment corresponding breeding program. Since the high throughput that now makes it possible to generate cultivar specific transcriptome data, improvement of aromatic rice exploring RNA-seq analysis is one of the best options to study differential gene expression ability to combat stress in diverse genotypes of available aromatic rice cultivars [5]. Further screening is necessary for successful breeding program. In West Bengal, India, though approximately 70 indigenous aromatic rice cultivars (IARCs) are in practice, but their improvement through screening the best genotypes for breeding program using high throughput technologies are lacking [6].

Our previous study was undertaken to screen some drought tolerant IARCs of West Bengal, India based on their redox-regulatory attributes at metabolic interface [7, 8]. In this regard, the selection of the redox-metabolic and transcriptomic data for judicious screening of the germplasms of some IARCs of West Bengal for their drought tolerant attributes found to be extremely significant, though not always decisive in plant stress biology research [9-12]. Further, the work will also help us to identify and ascertain whether, the redox regulated traits or standardized redox biomarkers can be targeted by plant breeders for selection or genetic manipulation for improving the ability of the aromatic rice cultivars to grow under drought stress.

One of the important approaches for understanding molecular insight or genetic regulatory mechanism into the extremely complicated regulatory signaling network including the redox signaling under abiotic stress involves comparative transcriptomic analysis in model plants. Both in *Arabidopsis* and rice, availability of complete reference genome enable us for sequencing their transcripts. Several workers in recent time, exploring transcriptome analysis, have revealed some important signaling and regulatory episodes under drought stress in both *Arabidopsis* and rice. For example, comparative transcriptomic investigation with RNA-seq analysis, in *Arabidopsis* has revealed the roles of some important transcription factors like WRKY70, WRKY46 etc. in up-regulating brassinosteroid mediated gene expression under drought stress [13]. Other workers [14] through their RNA sequence study also deciphered the roles of different hormonal signaling pathways [abscisic acid (ABA), jasmonic acid (JA), salicylic acid (SA), indole-3-acetic acid (IAA), gibberellic acid (GA) etc.] controlling metabolism during moderate drought stress of *Arabidopsis*. Xiong *et al* (2018) through their RNA sequencing and candidate gene analysis indicated the role of redox signaling under drought stress in rice [15]. Wang *et al* (2018) unfolded regulatory pathway involving ATBG1 and SYPCYP707A1/3 under drought stress in *Arabidopsis* [16]. Very recently other workers initiated some work towards understanding regulatory mechanisms associated with different magnitude of drought stress in rice through RNA-seq analysis. Bang *et al* (2019) characterized drought induced genes like DHHC4, AA4-type ATPases, CASPL5B1, modulin proteins etc. that regulate stomatal conductance, lignin deposition as contrivances of drought tolerance in rice [17]. Other important transcription factors, like NAC that exhibits up-regulation under drought and regulate various redox events necessary for drought tolerance is also identified in rice by transcriptome analysis [18].

All these transcriptomic studies made so far in recent times exhibited significant implication of the roles of specific genes and redox-regulatory genes in particular in drought stress tolerance. Here, in this study, we made an attempt with comparative analysis of changes in steady state level of transcript abundance through RNA sequencing of the redox competent and redox incompetent experimental IARCs identified through investigation of redox biology and physiological phenotyping of PIDS-raised seedlings and integrating them with redox metabolic and physiological phenotypes. In fact, this system biology approach of comprehensive physiological, metabolic and molecular regulatory mechanism regulating life processes like germination under drought might help us to identify the reliable traits and candidate genes for further breeding and transgenic experiments.

In this context, the present investigation of RNA-seq based comparative transcriptomic analysis was undertaken for two IARCs (cultivars Badshabhog and Jamainadu) previously screened on the basis of their redox-regulatory attributes under drought stress. Exploring the roles of differentially expressed genes under drought stress in two contrasting IARCs might help us to unravel the metabolic pathways and signaling molecules that are associated with drought tolerance. Further, the work will also help to understand the transcriptional regulation of oxidative stress under drought for two contrasting genotypes of IARCs with distinct redox-regulatory properties at metabolic interface.

Results

Polyethylene glycol (PEG) induced post-imbibitional dehydration stress (PIDS) modulates ROS-antioxidant interaction dynamics and sensitive redox biomarkers of germinating tissues of two contrasting indigenous aromatic rice cultivars (IARCs)

Previous experiments from this laboratory revealed significant differences in redox regulatory properties (modulation of ROS-antioxidant interaction status and sensitive metabolic redox biomarkers) between drought stress-raised IARCs Jamainadu and Badshabhog. When compared between these two experimental land races of aromatic rice, commonly cultivated in West Bengal, a clear difference in redox regulatory mechanisms have been revealed. Estimation of the components of the Halliwell-Asada pathway (both the redox metabolic turnover dynamics of ascorbate and glutathione and the activities of ascorbate peroxidase, dehydroascorbate reductase and glutathione reductase) revealed significant differences in processing of hydrogen peroxide (H_2O_2) between the experimental IARCs. Badshabhog exhibited better regulation of the pathway through enhanced turnover dynamics of redox components (ascorbate and glutathione) of the pathway through maintenance or up-regulation of the enzymes involved under drought (Fig 1).

[Insert Fig 1 here]

Comparatively better H_2O_2 processing ability in the cultivar Badshabhog as compared to the cultivar Jamainadu under identical condition of post-imbibitional dehydration stress (PIDS) through maintenance of ascorbate-glutathione cycle can be corroborated from the studies of in situ localization of H_2O_2 (through laser confocal microscopy; Fig 2A and Fig 2B and TMB/ 3,3',5,5'- tetramethylbenzidine staining; Fig 2C and Fig 2D). Nitroblue tetrazolium (NBT) staining for localizing superoxide (precursor of H_2O_2) also revealed the same trend of result (Fig 2E and Fig 2F).

157 **[Insert Fig 2 here]**

158

159 Further, standardization of sensitive redox biomarkers of oxidative stress of germinating
160 seeds of experimental IARCs grown under drought stress revealed significant changes of all
161 the parameters [superoxide, hydrogen peroxide, DCFDA (2', 7'-dichlorofluorescindiacetate)
162 oxidation, DPPH (2, 2$^{/}$-diphenyl-1-pycryl hydrazyl) radical scavenging properties, activity of
163 lipoxygenase and accumulation of membrane lipid and protein oxidation products
164 (hydroperoxide, conjugated diene, thiobarbituric acid reactive substances and free carbonyl
165 content)] under PIDS (Fig 3). However, when compared between these two, the PIDS-raised
166 seedlings of cultivar Badshabhog in general exhibited significantly lesser magnitude of
167 oxidative deterioration under PIDS as compared to Jamainadu (Fig 3). There was significant
168 level of regulation of accumulation of total and individual ROS (DCFDA oxidation,
169 superoxide and hydrogen peroxide accumulation) in the PIDS-raised seedlings of cultivar
170 Badshabhog through down regulation of lipoxygenase activity and comparatively better
171 radical scavenging property exhibited significant reduction of oxidative deterioration of
172 membrane protein (free carbonyl content) and lipid (TBARS, hydroperoxide and conjugated
173 diene) (Fig 3).

174

175 **[Insert Fig 3 here]**

176

177 In fact, the extent of lipid peroxidation and protein oxidation, the two sensitive redox
178 biomarkers of drought stress, showed significant differences between PIDS-raised IARCs,

Badshabhog and Jamainadu. There is in general, 414.99%, 110.71%, 508.04%, 174.05% more accumulations of hydroperoxide, TBARS, conjugated diene and free carbonyl content in the PIDS-raised seedlings of redox incompetent cultivar Jamainadu *vis-a-vis* the redox competent IARC Badshabhog.

The physiological phenotype assessed in terms of germination and early growth performences also strongly vouch the redox-regulatory properties of the experimental IARCs under drought. All the germination and early growth performance parameters (T_{50} value, vigor index, relative germination performance, relative growth index, relative water content, co-efficient of velocity of germination, germination rate index, mean germination time and mean daily germination) strongly vouch better germination performance for the cultivar Badshabhog *vis-a-vis* the cultivar Jamainadu grown under the same magnitude of PEG-6000 madiated PIDS (Fig 4).

[Insert Fig 4 here]

Comparative Transcrpitomic investigation (RNA-seq analysis) of post-imbibitional dehydration stress (PIDS)-raised redox competent and incompetent IARCs (Badshabhog and Jamainadu) using Illumina platform and their functional annotation

To explore the molecular mechanism and genetic regulation of germination influenced by post-imbibitional dehydration stress (PIDS), RNA-seq analysis of the germinating seedlings of most tolerant and susceptible indigenous aromatic rice cultivars (IARCs) were conducted to generate the global transcriptome profiles. We compared the transcriptome profiles of

PIDS-raised seedlings of the IARCs Jamainadu and Badshabhog (the most drought sensitive and resistant genotypes respectively, as screened through redox metabolic and physiological investigation) (Fig 5 and Table 1). A total number of 4 libraries were constructed and analysed for each sample (approximately 18-20 million of total reads generated for each sample) (Fig 6, Fig 7 and Table 2) and mapped to rice genome (http://plants.ensembl.org/Oryza_indica/info/index) using HISAT2. Subsequently, the map files were processed via feature counts. The unity mapped reads for each replication total around 18-20 million (Table 5) and there were 16-18 million total mapped reads for PIDS-raised IARCs, Jamainadu and Badshabhog respectively. Further, these were processed using features count for the determination of normalize expression level as the fragments of each transcript with per kilo base of transcript length per million mapped reads.

Total RNA was subjected to pair-end library preparation with Illumina TruSeq Stranded mRNA Library Preparation Kit. The mean sizes of the libraries are 292bp and 300bp respectively for samples PIDS (-1.619 MPa)-raised seedlings of IARC, Jamainadu (SBND1) and PIDS (-1.619 MPa)-raised seedlings of IARC, Badshabhog (SBND2). The libraries were sequenced using 2x150bp PE chemistry on Illumina platform for generating ~3 GB data per sample.

[Insert Fig 5 here]

Table 1: Quantification using Qubit® 2.0 Fluorometer

SAMPLES *[PIDS (-1.619 MPa)-raised seedlings of IARCs]*	*Concentration (ng/µl)*	*Yield (ng)*	*Remarks*
Jamainadu	**410**	**11.4**	**QC Pass**
Badshabhog	**520**	**14.5**	**Partially degraded**

[Insert Fig 6 & Fig 7 here]

Data generation

The next generation sequencing for all 2 samples [PIDS (-1.619 MPa)-raised seedlings of IARCs, Jamainadu and Badshabhog] were performed using Illumina Platform. The data statistics of High quality data is provided in the below table.

Table 2: HQ data statistics

Samples *[PIDS (-1.619 MPa)-raised seedlings of IARCs]*	*Total reads* *(PE)*	*Total Reads* *(R1+R2)*	*Total no of bases (R1+R2)*	*Data* *(Gb)*
Jamainadu	9,413,217	18,826,434	2,715,971,890	2.7
Badshabhog	10,330,002	20,660,004	2,990,081,749	3

Bioinformatics workflow

Reference guided transcript assembly was performed for all the samples, first by mapping HQ reads on reference genome using hisat2 (v 2.2.1) and then performing transcript assembly by StringTie (v 2.1.4). A consensus set of transcripts was obtained using SringTie merge function which merges together all the gene structures found in any of the samples. Transcript abundance was then estimated using merged transcript consensus again using StringTie and read counts thus obtained for each transcript were taken as input for differential expression analysis using edgeR package. Gene ontology (GO) and pathway analysis of the differentially expressed transcripts were performed using UniprotKB and KEGG-KAAS (Kyoto encyclopedia of genes and genomes-KEGG automatic annotation server) server respectively.

Reference genome information

The reference genome of *Oryza sativa* Indica Group and its corresponding GTF file was downloaded from ensembl database (http://plants.ensembl.org/*Oryza*_indica/info/index) (Table 3). The genome of *Oryza sativa* is of ~ 374Mb.

Table 3: Reference genome statistics

Description	***Stats***
# Chromosomes	12
Total genome length (bp)	374,545,499
Mean sequence size (bp)	31,212,124
Max sequence size (bp)	47,283,185
Min sequence size (bp)	21,757,032

The reference GTF (gene transfer format) file contains genome annotation. It includes information (locus and description) regarding genes and proteins. There are a total of 38,676 genes as per the GTF file of ensembl's *Oryza sativa* reference genome (Table 4).

Table 4: Detail Information of GTF file of ensembl's *Oryza sativa* L. Indica genome

Genome elements	***# sequences***
gene	38,676
CDS	165,685
exon	166,492
Start codon	37,878
Stop codon	37,878
Transcript	38,676

Mapping to reference genome

Before mapping, indexing of the reference genome was carried out using hisat-build (HISAT2 specific indexer program). Then the input reads, in FASTQ format, were given to HISAT2 aligner along with the reference genome index for alignment. Thus analysis begins by mapping HQ reads against the *Oryza sativa* Indica, reference genome using HISAT2 to create alignment in SAM/BAM format for each sample. This is to identify the positions from where the reads originated. This mapping information allows us to collect subsets of the reads corresponding to each gene, and then to assemble and quantify transcripts represented by those reads. The mapping statistics are provided in the table 5

Table 5: Reads Mapping Statistics

Samples *[PIDS (-1.619 MPa)-raised seedlings of IARCs]*	*Total Reads (R1+R2)*	*No. of mapped reads*	*% of mapped reads*
Jamainadu	18,826,434	16,890,240	89.72%
Badshabhog	20,660,004	18,436,279	89.24%

Transcript assembly

StringTie assembles transcripts from RNA-seq reads that have been aligned to the genome, first grouping the reads into distinct gene loci and then assembling each locus into as many isoforms as are needed to explain the data. Following this, StringTie simultaneously assembles and quantify the final transcripts by using network flow algorithm and starting from most highly abundant transcripts. The reference genome GFF (general feature format) annotation files, containing exon structures of "known" genes, are then used to annotate the assembled transcripts and quantify the expression of known genes as well derive clues if a novel transcript has been found in the sample.

After assembling each sample, the full set of assemblies is passed to StringTie's merge function, which merges together all the gene structures found in any of the samples. This step is required because transcripts in some of the samples might only be partially covered by reads, and as a consequence only partial versions of them will be assembled in the initial StringTie run. The merge step creates a set of transcripts that is consistent across all samples, so that the transcripts can be compared through some important steps. The statistics of merged transcripts and individual transcript assembly is provided in the table 6.

Table 6: Statistics of transcript assembly

Sample Name	*# Assembled Transcripts*
Merged GTF	55,886
PIDS (-1.619 MPa)-raised seedlings of IARC, **Jamainadu**	37,051
PIDS (-1.619 MPa)-raised seedlings of IARC, **Badshabhog**	37,972

The output consists of assembled gene/transcript GTF file for all samples and another GTF file resulting from the merge step are as described above.

Comparison of assembled transcripts with reference transcripts

For comparison of assembled transcripts with reference transcripts, Gffcompare utility was run taking the reference GTF and the string-tie merged GTF file. This produces an output file, which adds to each transcript a "class code" and the name of the transcript from the reference annotation file to check how the predicted transcripts relate to an annotation file. Meaning of each class code is depicted in figure 8. Potential novel isoform (Class code "j") that shares at least one splice junction with a reference transcript were extracted. A total of 7,739 novel isoforms with the class code "j" were identified.

[Insert Fig 8 here]

308 ***Differential expression analysis***

309 Abundances of the merged transcripts in all the 2 samples were estimated using StringTie. A
310 python program (prepDE.py) was used to extract the read count information directly from the
311 files generated by StringTie.

312 For differential expression analysis, sample comparison was made as shown in table 7.

313 **Table 7:** Sample comparison for differential expression analysis

Sample SBND1	***Sample SBND2***
PEG-6000 induced seedlings of IARC, **Jamainadu**	PEG-6000 induced seedlings of IARC, **Badshabhog**

314

315 Differential gene expression (DGE) was inferred between sample groups by applying the R
316 package edgeR (v3.28.1). edgeR is a bioconductor package based on negative binomial
317 distribution method. The analysis provides tabular result and the description of each column
318 is provided in the table 8.

319 **Table 8:** Column description of DESeq output

Column Name	***Feature identifier***
CPM(control)	Reads per million mapped reads or Counts per million mapped reads (CPM) of Control sample
CPM(Test)	Reads per million mapped reads or Counts per million mapped reads (CPM) of Treated sample
log2FoldChange	the logarithm (to basis 2) of the fold change(treated/control)
logCPM	Log10 of CPM value
pval	pvalue for the statistical significance of this change
FDR	FDR adjusted pvalue (q-value)

320

PIDS-raised DEG (differential expression of genes) of seedlings of Jamainadu –Vs- PIDS-raised seedlings of Badshabhog

The criterion used to identify up-regulated and down-regulated transcripts along with the significance is provided in the table 9

Table 9: Criteria used to identify up-regulated and down-regulated genes and assigning the significance

Condition	***Status***
log2FC > 0	Up regulated
log2FC < 0	Down regulated
log2FC > 0 and q-value <0.05	Significantly up regulated
log2FC < 0 and q-value <0.05	Significantly down regulated

Differential expression analysis statistics is provided in table 10.

Table 10: Differential gene expression statistics

Combination	***Total Differentially expressed gene***	***Down-regulated***	***Up-regulated***	***Significant Down-regulated***	***Significant Up-regulated***
PIDS-raised seedlings (-1.619 MPa) of IARCs, **Jamainadu** -Vs- **Badshabhog**	32,988	16,177	16,811	3,482	3,866

The Venn diagram representing number of genes expressed in both the seedlings of experimental IARCs raised from PIDS and uniquely up-regulated (exclusively expressed) genes is given below (Fig 9):

[Insert Fig 9 here]

Heat map for significantly expressed differential genes in combination

List of transcripts were selected for heatmap generation based on following criteria

- Significantly expressed transcripts present in both the samples and highly regulated in terms of log2FoldChange (top 50 transcripts).
- Transcripts must have proper annotations.

pheatmap package from R software was used for producing heatmap. The color coding ranges from red to blue where shades of red represent high transcripts expression and shades of blue represents low transcript expression (Fig 10).

[Insert Fig 10 here]

350 ***MA for significantly expressed differential genes in combination***

351 The plot visualizes the differences between measurements taken in two samples, by
352 transforming the data onto M (log ratio) and A (mean average) scales, then plotting these
353 values (Fig 11).

354

355 **[Insert Fig 11 here]**

356

357 ***Volcano plot for significantly expressed differential genes in combination***

358 The "volcano plot" arranges expressed genes along dimensions of biological as well as
359 statistical significance (Fig 12).

360

361 **[Insert Fig 12 here]**

362

363 ***Gene Ontology (GO) analysis***

364 The Gene Ontology data provides controlled defined terms representing gene product
365 properties. These cover three domains: Cellular Component, the parts of a cell or its
366 extracellular environment; Molecular Function, the elemental activities of a gene product at
367 the molecular level, such as binding or catalysis; and biological process, operations or sets of
368 molecular events with a defined beginning and end, pertinent to the functioning of integrated
369 living units: cells, tissues, organs, and organisms. For obtaining gene ontology for

differentially expressed transcripts, they were first annotated against Uniprot database followed by mapping against UniprotKB. The GO domain distribution is shown below (Table 11 and Fig 13).

Table 11: GO Distribution of Differential expression transcripts

Combination	***# Total DEG***	***Cellular Component***	***Biological Process***	***Molecular Function***
PIDS-raised (-1.619 MPa) seedlings of **Jamainadu** -Vs- PIDS-raised (-1.619 MPa) seedlings of **Badshabhog**	32,988	20,942	19,412	19,909

[Insert Fig 13 here]

The comparative Gene ontology (GO) of both PIDS-raised experimental IARCs Badshabhog and Jamainadu considering 7348 DEGs [that includes genes expressed in both the land races as well as uniquely up-regulated genes (q-value <0.05)] are given in figure 14A and 14B.

[Insert Fig 14 here]

From this status of DEG, further, genes are taken which are uniquely up-regulated in either experimental IARCs Jamainadu or Badshabhog (i.e., present exclusively in both the experimental land races). Such exclusively up-regulated genes for Jamainadu and Badshabhog are 1741 and 2109 respectively (Fig 15A and 15B).

388 **[Insert Fig 15 here]**

389

390 ***Pathway analysis***

391 Ortholog assignment and mapping of the differentially expressed transcripts to the biological
392 pathways were performed using KEGG automatic annotation server (KAAS). Differentially
393 expressed transcripts were compared against the KEGG database using BLASTX with
394 threshold bit-score value of 60 (default). Pathway analysis was performed using all
395 differentially expressed transcripts. The mapped transcripts represented metabolic pathways
396 of major biomolecules such as carbohydrates, lipids, nucleotides, amino acids, glycans,
397 cofactors, vitamins, terpenoids, polyketides, etc. The mapped transcripts also represented the
398 genes involved in metabolism, genetic information processing, and environmental
399 information processing and cellular processes (Table 12 and Fig 16).

400

401 **[Insert Table 12 and Fig 16 here]**

402

403 Apart from plant hormone signal transduction KEGG pathways that have depicted with one
404 gene entry or functional unit of the pathway, separate pathways for both the experimental
405 land races raised from PIDS for uniquely up-regulated genes (1741 genes for Jamainadu –Vs-
406 2109 genes of Badshabhog) with q-value <0.05 is given in Table 13.

407

408 **[Insert Table 13 here]**

Further, Kyoto encyclopedia of genes and genomes (KEGG) analysis to characterize and identify enriched pathways contributed by drought responsive DGE in both the experimental IARCs, it was revealed that apart from metabolism (the most significantly enriched pathways), environmental information processing and genetic information processing constitute the core DEGs in these pathways for the experimental IARCs, indicating the roles of signaling pathways and gene expression reprogramming and regulation in drought tolerance of both the experimental IARCs.

Discussion

The selection of physiological, metabolic and molecular markers for judicious screening of germplasms of rice for their drought stress tolerance is extremely significant, though not always a decisive approach in agricultural research. The integration of 'omic' approaches, as in marker assisted selection along with important metabolic and biochemical data always ensures a system level information of the complex drought response necessary for tolerance [10, 19, 20]. In this aspect, the effectiveness of drought induced oxidative stress tolerance through redox regulation may be used as quality traits for standardizing biomarkers of drought stress tolerance [21-25]. In this background, the present work made an effort to assess and standardize some important redox metabolic and molecular parameters along with their physiological phenotyping for screening some IARCs commonly cultivated in West Bengal, India. In this regard, the integration of redox metabolic and physiological data along with genomic data of drought induced IARCs ensues a better understanding of complex redox-regulatory traits of plants in conferring drought tolerance. The present work, in this perspective, explores the redox biology of PIDS induced seedlings of two important IARCs of West Bengal which not only help us to elucidate the complex redox-regulatory

mechanisms necessary for drought tolerance but may also be used as stress tolerance biomarkers for screening drought resistant germplasms of aromatic rice.

Two contrasting indigenous aromatic rice cultivars (drought sensitive Jamainadu and drought tolerant Badshabhog) screened on the basis of comparative redox biology and physiological phenotyping were further investigated for unfolding genetic regulation of drought stress based on comparative transcriptomics using RNA-seq. Through RNA-seq analysis and subsequent functional annotation of the data, I have identified differential expression of genes between PIDS-raised seedlings of IARCs Jamainadu and Badshabhog. In order to reduce the artifacts, several filtering parameters were employed during data analysis. Genome reference sequence had been utilized to unfold the best impact on the downstream analysis such as number and variety of DEGs.

When we compared the differential expression of genes between PIDS-raised seedlings of Jamainadu and Badshabhog, a total of 32988 genes found to be differentially expressed. Further, when I compared between these two, 16177 genes found to be down-regulated in Badshabhog as compared to 16811 up-regulated genes. However, when the significant down-regulated and up-regulated genes were compared between PIDS-raised Jamainadu and Badshabhog, the number became 3482 and 3866 respectively. When the list of transcripts were selected for heatmap generation based on significantly expressed transcripts present in both the samples (highly regulated in terms of log2 fold change (log2FC) with proper annotations, 50 genes were highlighted which mainly include redox genes, GTPase activity protein, lysine specific demethylase, NADH-dehydrogenase, peroxisome biogenesis protein, GDSL-esterase/lipase transport protein ACC-61, ATP-dependent RNA helicase etc. So the genes commonly up-regulated in both the genotypes under water stress mainly involves redox

functions, signaling, membrane trafficking, replication and protein synthesis and metabolic role.

The genes otherwise differentially expressed significantly (highly expressed genes) under PIDS in redox competent IARC Badshabhog as compared to redox incompetent IARC jamainadu includes NADH-dependent malic enzyme isoform (MSTRG2338.1), SUMO activating enzyme subunit 2 isoform XI (MSTRG.21850.1), 60S ribosomal protein (BGI Os GA 001808-TA), peroxysome biogenesis protein isoform XI (BGI 0SGAO28937TA), NADH dehydrogenase (Ubiquinone) 1 beta complex subunit (BG10SGA033496-TA), RAS related protein RABE1C (BG10SGA013829-TA), lysine specific demethylase SE14 like isoform (MSTRG.10912.2), stromal cell derived factor 2 like protein (MSTRG.23102.1) and hypothetical protein OSI_38644 (BGI0SGA036042-TA), hypothetical protein Osl_12555, hypothetical protein 32602. The other genes which got significantly down-regulated in PIDS-raised seedling of IARC Badshabhog in comparison to the cultivar Jamainadu (where these genes got significantly expressed) include eukaryotic translation initiation factor JA-2polyubiquitin A, 60S ribosomal protein, hypothetical protein CR513_38538, FT interacting protein 1, GDSL esterase/ lipase LTL1. The genes which got highly expressed in Badshabhog are found to be mainly concerned with CO_2 concentrating mechanism (NADH-dependent ME), peroxysomal biogenesis, protein modification, protein synthesis (60S ribosomal protein), mitochondrial ETC functioning (NADH dehydro ubiquinone), intercellular protein transport (rabc1-RAS related protein), histone demethylation associated with developmental process (lysine specific demehylase), regulation of apoptosis (stromal cell related factor 2 like protein). So, deep dissection of significantly over-expressed protein in redox-competent IARC Badshabhog revealed their better cellular and molecular activities

along with metabolic regulation to cope up with drought stress, when compared to the IARC Jamainadu [26-30].

Other genes, though not expressed significantly but exhibited marginal up-regulation in the IARC Badshabhog as compared to Jamainadu includes redox genes [L-ascorbate oxidase/ peroxidase, monothiol glutaredoxin-S1, thioredoxin-like protein AAED1 (chloroplastic), thioredoxin-like protein CXXS1, NADH-dehydrogenase (ubiquinone)-1-beta subcomplex subunit 3-B, NADH-dehydrogenase subunit 6 and K, lipoxygenase 6 isoform-XI etc.], signal transduction related genes (dehydration-responsive element-binding protein 1I, auxin-related protein 1 isoform X1, auxin efflux career component 3a isoform X1, auxin response factor 16 isoform X1, auxin-responsive protein SAUR 19, SAUR36, SAUR77, ethylene-responsive transcription factor 5, ethylene-responsive transcription factor ERF177, Ca^{2+}-dependent protein kinase12, 17, 4 isoformX1, calmodulin-binding protein 60 B isoform X1, MAP kinase14, MAP kinase kinase kinase 1, MAP kinase kinase kinase ANP1, serine/threonine-protein kinase OsK4, BSK2, D6PK etc.), transcription factor related genes (MybS1, NAI1, WRKYtranscription factor 32, bZIP transcription factor 16 isoform X2, bZIP transcription factor TRAB1, ethylene responsive transcription factor 5, transcription factor BHLH113, BHLH18, BHLH79 etc.), replication and protein synthesis related genes (40S ribosomal protein S11, S18, S24-1, S-9; 60S ribosomal protein L18a, L24, L26-1,L29-1, ATP-dependent helicase BRM, ADP ribosylation factor 2, DNA-directed RNA polymerase I, DNA repair protein XRCC4 etc.) transporter related genes [ABC transporter B family member 28, 13, 37, 39, 41, 45, K-transporter1, K(+) efflux antiporter 4, amino acid permease BAT1, amino acid permease 3 and 4] and metabolic genes [α-amylase/trypsin inhibitor, anthocyanin reductase (2S)-flavan-3-ol-forming, probable chalcone-flavonone isomerase 3, ART1,

chalcone-flavonone isomerase, cysteine synthase, cysteine protease, isoflavone 3'-hydroxylase isoform X1, isochorismate synthase 1 etc.].

So, the comparison of transcript between PIDS-raised seedlings of Jamainadu and Badshabhog suggests that there is a major difference in the number of genes expressed under identical condition of PIDS. Jamainadu, the drought susceptible IARC (screened on the basis of investigation of redox biology and physiological phenotyping), exhibited a moderate transcriptional response when exposed to water stress. While the drought tolerant IARC, Badshabhog (screened on the basis of investigation of redox biology and physiological phenotyping) showed the modest transcriptional expression. These findings potentially show that the cultivar Badshabhog is relatively transcriptionally stable under dehydration stress as compared to Jamainadu. This result is in concurrence with the previous result of Yang *et al* (2017) and Ereful *et al* (2020) [28, 30].

Functional annotations of transcript data involving gene ontology (GO) provide information on potential functions of genes as well. For obtaining gene ontology for differentially expressed transcripts, they were first annotated against uniprot database following mapping against uniprotKB. GO domain distribution showed that out of 32988 differentially expressed transcripts in PIDS-raised seedlings of Jamainadu and Badshabhog, 20942 belongs to cellular component, 19412 belongs to biological process, 19909 belongs to molecular function. So far as molecular function is concerned it was estimated through GO distribution of DEG that almost 1% of differentially expressed genes in drought susceptible IARC Jamainadu and drought resistant IARC Badshabhog belongs to antioxidant activity. Other notable molecular function related to differentially expressed transcripts are transcriptional and translational regulatory activity, important cellular processes, biogenesis of important cellular component and carrier activity etc. The GO distribution for biological processes that showed the

differential expression of transcripts between Jamainadu and Badshabhog mainly involves signaling, regulation of biological processes, metabolic processes, response to stress/stimuli, positive and negative regulation of metabolic processes etc. The percentage of genes involved in antioxidant functioning, cell signaling and regulation of biological processes also accounts for more than 1% percentage of genes. So, the IARC, Badshabhog exhibited greater GO distribution so far as cell signaling antioxidant, biological and metabolic regulation, transcriptional and translational regulation etc. are concerned. Similar kind of results has been noticed by Toni *et al* (2017) [31] and Ereful *et al* (2020) [30]. So, the comparison of transcript between PIDS-raised seedlings of Jamainadu and Badshabhog suggests that there is a major difference in the number of genes expressed under identical condition of PIDS. Jamainadu, the drought susceptible IARC (screened on the basis of investigation of redox biology and physiological phenotyping), exhibited a moderate transcriptional response when exposed to water stress. While the drought tolerant IARC, Badshabhog (screened on the basis of investigation of redox biology and physiological phenotyping) showed the modest transcriptional expression. These findings potentially show that the cultivar Badshabhog is relatively transcriptionally stable under dehydration stress as compared to Jamainadu. This result is in concurrence with the previous result of Yang *et al* (2017) and Ereful *et al* (2020) [28, 30]. The significantly higher percentage of antioxidative genes, differentially expressed in Badshabhog as compared to Jamainadu corroborate the fact that the IARC Badshabhog is capable of scavenging more reactive oxygen species and restore redox homeostasis under similar kind of PIDS. Moreover the greater transcript abundance associated with signaling, biological regulation and response to stimulus for the PIDS-raised Badshabhog as compared to Jamainadu also supports the better preparedness of the IARC, Badshabhog under PIDS through cell signaling and metabolic adjustment [30, 32].

549 Ortholog assignments and mapping of differentially expressed transcripts to the biological
550 pathways were performed using KEGG automatic annotation server (KAAS). Subsequently
551 the differentially expressed transcripts were compared against KEGG database, BLASTX
552 with threshold bit-score value. Here the map transcripts represented metabolic pathways of
553 major biomolecules as well as environmental regulation of those pathways and cell signaling.
554 The growth signaling KEGG pathways, that has been assigned to at least one gene entry or an
555 ortholog table entry that correspond to a gene that is part of the functional unit of the pathway
556 showed several signal transduction pathway being influenced by drought stress. The most
557 important of which are abscisic acid, brassinosteroid, salicylic acid, jasmonic acid and
558 ethylene. The role of these hormonal pathways in one way or another is influenced by the
559 redox status of the cells. So, the differences in redox-regulatory properties under PIDS
560 between the cultivars Jamainadu and Badshabhog seems to have differential impact on the
561 transcript abundance of some of the genes associated with these signaling molecules. In fact,
562 the biosynthesis of metabolites like phenylalanine, linoleic acid, brassinosteroid, cysteine,
563 methionine and carotenoids seems to have profound impact on the metabolism and signaling
564 of salicylic acid (SA), jasmonic acid (JA), brassinosteroid (BS), ethylene and abscisic acid
565 (ABA) respectively. Badshabhog in general showed significant up-regulation of important
566 genes of these metabolic pathways. ABA, JA, SA and ethelyne play key role in responding to
567 environmental stresses [33]. Using KEGG pathway analysis, it was found that both the
568 IARCs, Jamainadu and Badshabhog found to have impact on these hormonal pathways and
569 signaling but when compared the tolerant IARC Badshabhog seems to have better response
570 for drought tolerance.

571

572

Conclusion

The overall result of comparative transcriptomic investigation suggests that IARCs Badshabhog and Jamainadu explore varying strategies to deal with PIDS. The cultivar Badshabhog exhibited a significantly greater molecular reprogramming presumably more through up-regulation of metabolic and energy demanding processes along with implementing better signaling strategies to deal with drought stress during early germination. The defensive strategies as adopted through antioxidative defense mechanism, DNA repair and transcriptional regulation are also seems to be the better adopted by IARC Badshabhog *vis-a-vis* the cultivar Jamainadu. The signaling pathway including ABA, JA, SA, ethylene and IAA which either individually are in association with redox cue were shown to be significantly up-regulated in Badshabhog. The controlled redox-regulatory event that generates conducible internal redox cue in PIDS-raised IARC Badshabhog might have augment hormonal signal transduction pathways, receptor activity and metabolic reprogramming associated with drought tolerance. Though both the experimental IARCs employ genetic regulation through overall metabolic processes, regulation of transcription, ion balance, programmed cell death etc. for survival, but the cultivar Badshabhog showed enhanced transcriptional regulation associated with peroxisomal and chloroplastic pathways, cell signaling, defensive processes under drought stress during early germination as compared to cultivar Jamainadu. These findings necessitates further dissection of this drought induced attributes in the screened drought tolerant IARC Badshabhog. So, the present study clearly revealed molecular basis of metabolic competence including redox-regulatory properties of two contrasting IARCs under drought stress. The information generated from this comparative transcriptome analysis along with their sensitive redox-metabolic data will definitely assist rice breeders in breeding program for augmenting the productivity of IARCs.

Methods

Collection of experimental germplasms of indigenous aromatic rice cultivars (IARCs) and their maintenance in Crop Research and Seed Multiplication Farm (CRSMF), The University of Burdwan

Seeds of two experimental indigenous aromatic rice cultivars [IARCs *Oryza sativa* L., Cultivars Jamainadu and Badshabhog, commomnly cultivated in different areas of West Bengal)], were collected from Chinsurah Rice Research Station, Chinsurah, Government of West Bengal, India and the germplasms were multiplied and maintained at Crop Research and Seed Multiplication Farm (CRSMF), The University of Burdwan, Burdwan, West Bengal, India.

Treatment of PEG-6000 to induce post-imbibitional dehydration stress (PIDS)

Seeds of the two indigenous aromatic rice cultivars [IARCs (*Oryza sativa* L., Cultivars Jamainadu and Badshabhog)], selected as experimental material, have been collected from Chinsurah Rice Research Station, West Bengal, and later grown in CRSMF for three successive years. Seeds of the experimental cultivars grown at CRSMF, were washed with distilled water and were treated with 0.2% $HgCl_2$ for 5 minutes and then washed thrice with sterile distilled water. The surface sterilized seeds were imbibed in distilled water for 48 hours in darkness at $25^o \pm 2^oC$ and thereafter, were sown on moist filter paper in petri plates and were placed in standardized conditions of thermostat-controlled seed germinator cum stability chamber, maintained at $25^o \pm 2^o$ C temperature. For imposing post-imbibitional dehydration stress, water-imbibed seed lots of each cultivar were treated with -1.619MPa PEG-6000 for 7 days, with intermittent change of treating solutions in petri plates. Dose of PEG-6000 for imposing PIDS were standardized based on initial pilot experiment. For

620 untreated control set, water imbibed seeds were sown directly in petri plates on moist filter
621 paper. All the seed lots were allowed to grow at 25°±2°C with 14 hour photo period (light
622 intensity 270 µmol m^{-2} s^{-1}) and 78±2% relative humidity. For all biochemical analysis 168
623 hours old seedlings raised from aforesaid conditions were used.

624 **Assessment of ROS-antioxidant interaction dynamics**

625 ***Efficiency of Halliwell-Asada pathway***

626 ***Extraction and estimation of associated antioxidant enzymes (ascorbate peroxidase,***
627 ***dehydroascorbate reductase and glutathione reductase)***

628 Ascorbate peroxidase (APOX, EC 1.11.1.11) activity was determined according to Nakano
629 and Asada (1981) [34]. Homogenate was prepared by homogenizing the tissue (500 mg) with
630 5mL of potassium-phosphate buffer (pH-7.0) and it was centrifuged at 6000 rpm for 15
631 minutes to collect the supernatant (enzyme extract). The assay mixture containing 1 mL,
632 0.5mM ascorbic acid- potassium phosphate buffer (50mM, p^H-7.0) solution, 1 mL of H_2O_2
633 (0.1 mM) and 200 µL of enzyme extract was measured at 290 nm for the determination of
634 APOX by following the decrease in absorbance for 1 minute. The enzyme extract was added
635 lastly to start the reaction.

636 The estimation of dehydroascorbate reductase (DHAR, EC 1.8.5.1) activity was done by
637 following the process of Nakano and Asada (1981) with some modification [34]. 500 mg of
638 tissue was homogenized with 10 mL of 50mM potassium-phosphate buffer (p^H-7.0) and then
639 centrifuged at 5000 rpm for 15 minutes in 4^0C temperature). The enzyme extract was
640 collected as supernatant. The enzyme extract (1 mL) was mixed with 0.5 mL of 50 mM
641 potassium-phosphate buffer (pH- 7.0), 0.1 mL of 2.5 mM GSH, 0.2 mM DHA (0.2 mL) and

642 0.1 mM ethylenediamine tetra-acetic acid (0.1 mL) and reaction rates were measured by the
643 increase in absorbance at 265nm in 10 seconds and in 30 seconds after adding the enzyme.

644 Glutathione reductase (GR, EC 1.6.4.2) activity was measured according to Schaedle and
645 Bassham (1977) [35]. 500 mg of tissue was extracted with 10 mL, 50 mM potassium-
646 phosphate buffer (p^H-7.2) and centrifuged at 5000 rpm for 15 minutes. The enzyme extract
647 was collected as supernatant and reaction mixture was prepared. The reaction mixture was
648 contained 4 mL of 50 mM potassium-phosphate buffer (p^H-7.0), containing 2 mM Na2-
649 ethylenediamine tetra-acetic acid, 0.15mM NADPH, and 0.5 mM oxidized glutathione
650 (GSSG) and 100 μL homogenate (7 mg protein mL^{-1}). After 30 minutes of incubation
651 NADPH oxidation was followed at 340 nm. The actual activity was determined by
652 subtracting the correction factor (absorbance of assay mixture without NADPH at 340 nm
653 was taken) from the absorbance of reaction mixture. The enzyme activity in all cases was
654 expressed as enzyme unit g^{-1} dm min^{-1} according to Fick and Qualset (1975) [36].

655 ***Extraction and estimation of ascorbate and glutathione***

656 1 g tissue was homogenized in 10 mL cold 5% metaphosphoric acid. After centrifugation at
657 15,000g for 30 min at 4°C, the supernatant was collected for analyses of ascorbate and
658 glutathione. This extraction procedure was little modified from the method given by Gossett
659 *et al* 1994 [37]. The measurement of total ascorbate and reduced ascorbate (AsA) contents
660 were modified from the method of Law *et al* (1983) [38]. Total ascorbate contents were
661 determined in a 3 mL mixture. Enzyme extract (0.3 mL) was mixed with 0.15 mL10 mM
662 dithiothreitol and 0.75 mL 150 mM phosphate buffer (pH 7.4) containing 5 mM
663 ethylenediamine tetra-acetic acid and was incubated at 25°C for 10 min, followed by addition
664 of 0.15 mL, 0.5% of N-ethylmaleimide. Then 10% trichloroacetic acid (0.6 mL), 44%
665 orthophosphoric acid (0.6 mL) and 0.6 mL of 4% of α,α′-bipyridyl were added. Finally, 3%

of $FeCl_3$ was added and the mixture was incubated in 40°C for 40 min and the absorbance was detected at A_{525}. AsA contents were determined by adding distilled water instead of dithiothreitol and N-ethylmaleimide and then followed the same method as above. Total and reduced contents were estimated from the standard curve of 0-100 µg mL^{-1} L-AsA determined by the above method. Dehydro ascorbate (DHA) contents were calculated by the subtraction of AsA from total AsA.

Total glutathione contents were determined by the change in absorbance at 412 nm for 1 minute, according to the method reported by Zhang and Kirkham, 1996 [39]. The assay mixture contained 20 µL of distilled water, 150 µL of supernatant, 700 µL of 0.3mM NADPH, 100 µL of 6 mM DTNB (dithiobis-2-nitrobenzoic acid) and 50 µL of glutathione reductase (GR). The contents of glutathione (reduced form) were estimated from the standard curve of 0-30 µmol mL^{-1} glutathione. After the removal of glutathione (GSH) by 2-vinylpyridine derivative (20 µL), glutathione disulfide (GSSG) contents were determined, and the glutathione (GSH) contents were calculated by the subtraction of glutathione disulfide (GSSG) contents from total glutathione contents.

In situ staining for visualization of superoxide and hydrogen peroxide in seedlings of IARCs exposed to PIDS

For the detection of superoxide and hydrogen peroxide the process of He *et al* (2009) was followed [40]. In case of superoxide the seeds of different stressed conditions as well as control set were incubated separately in 6 mM nitroblue tetrazolium in 10 mM TRIS-HCl buffer (p^H-7.4) at room temperature for 15 minutes. The accumulation of superoxide anion was detected by observing the dark blue colour as compared to untreated control set. Hydrogen peroxide was detected by soaking the stressed and untreated control seedlings in

689 0.42 mM TMB (3, 5, 3′5′-tetramethylbenzidine) solution in 15 mM TRIS-acetate buffer (p^H-
690 5) for 2 hours. Blue-green colour can be monitored to indicate the accumulation of H_2O_2.

691 ***In situ* localization of hydrogen peroxide by confocal microscopy in seedlings of indigenous**
692 **aromatic rice cultivars (IARCs) exposed to post-imbibitional dehydration stress (PIDS)**

693 The sample preparation was done by following the method of Kaur *et al* 2016 [41]. Root of 7
694 days old seedlings of experimental rice cultivars were dipped immediately in 10µM
695 H_2DCFDA solution and kept at room temperature. After 2 hours samples were washed thrice
696 with autoclaved milliQ water and slides were prepared with 20% glycerol. Aaccumulation of
697 H_2O_2 in roots was identified by DCFDA staining and confocal microscopy using Leica
698 application suite X software (microscope model number was Leica TCS SP8, laser scanning
699 mode 488nm, emission at 505-530 nm, objective used was 20X) in differentially grown
700 seedlings (7 days old) raised from post-imbibitional dehydration stress (-1.619 MPa) *vis-a-*
701 *vis* their untreated control. Green fluorescence indicates presence of H_2O_2.

702 **Assessment of redox-biomarkers**

703 ***Estimation of total ROS generation***

704 Total ROS estimation was performed by an *in vitro* assay. Seedling tissue were placed (30
705 mg) separately in 8 mL of 100 µM 2', 7'-dichlorofluorescindiacetate (DCFDA, Sigma)
706 solution (in 40 mM TRIS-HCl buffer, pH-7.0) at 30^0C. After 60 min Supernatants were taken
707 and fluorescence was monitored in a spectrofluorometer (Hitachi, Model F-4500 FL
708 Spectrophotometer) with excitation at 504 nm and emission at 525 nm [42]. To differentiate
709 ROS from other long-lived substances able to react with DCFDA, additional controls were
710 performed. For additional controls, seedling tissues were incubated without DCFDA for 60
711 min and then tissues were removed followed by addition of DCFDA which is 60 min before

fluorescence was determined. This florescence values was subtracted from all readings to assess the fluorescence that depend on ROS. Corrections for auto-fluorescence were made by the inclusion in each experiment of parallel blanks, i.e., assay mixture without plant material.

Extraction and estimation of superoxide ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) generation

Superoxide was extracted and estimated by the process of Chaitanya and Naithani (1994) with some necessary modifications [43]. 500 mg of tissues was homogenized in cold with 5 mL of 0.2 M sodium phosphate buffer, (pH 7.2), with addition of diethyldithiocarbamate (10^{-3} M) to inhibit superoxide dismutase (SOD) activity. The homogenates was immediately centrifuged at 3000 g at 4^0C for 15 min. In the supernatant, superoxide anion was measured by its capacity to reduce nitroblue tetrazolium (2.5×10^{-4} M). The absorbance of the end product was measured at 540 nm. Formation of superoxide was expressed as $\Delta A_{540}\ g^{-1}$ dm.

In case of determination of hydrogen peroxide, the procedure of MacNevin and Uron (1953) was followed using titanic sulfate [44]. For this, 1 g of tissue was extracted with 5 mL of cold acetone and filtered through Whatman No.1 filter paper and volume made up to 10 mL with distilled water. Now 1 mL of 5% titanic sulfate (in 20% H_2SO_4) was added to this, which was followed by addition of 2 mL of concentrated NH_4OH and finally centrifuged at 6000 rpm for 10 minutes. Pellet obtained was washed with 5 mL of acetone (thrice) and then centrifuged at 5000rpm for 10 minutes. Then the pellet was dissolved in 3 mL of 2(N) H_2SO_4 and absorbance was taken at 420 nm against a blank.

Assessment of radical scavenging property or total antioxidant capacity (DPPH radical scavenging property)

For determination of DPPH (2, 2′-diphenyl-1-pycryl hydrazyl) free radical scavenging activity the process of Mensor *et al* (2001) was followed with little modification [45]. 1.5

gram of dry sample (seedling tissues kept at 45° C for two days) was extracted with 30 mL 80% methanol at 28° C for 24 hours in shaking incubator. Extracts were centrifuged at 3500 rpm for 20 minutes at 4° C. Supernatant was collected and filtered and filtrate was used for DPPH radical scavenging activity. For estimating the radical scavenging activity 1 mL sample was mixed with 3 mL DPPH (0.04 mg mL^{-1} ethanol) and incubated for 30 minutes in darkness and then absorbance was taken at 517 nm. Total antioxidant capacity (TAC) was calculated as:

$$TAC\ (\%) = \left[1 - \frac{Ai - Aj}{Ac}\right] \times 100$$

Where A_i= 1 mL sample + 3 mL DPPH; A_j = 1mL sample + 3 mL ethanol ; A_c= 1 mL ethanol + 3 mL DPPH

Extraction and estimation of thiobarbituric acid reactive substances

To estimate membrane lipid peroxidation, test for thiobarbituric acid reactive substances (TBARS) was performed using the procedure of Heath and Packer (1968) [46]. The membrane lipid peroxidation of drought-stressed as well as control tissues was estimated in terms of malondialdehyde accumulation. 200 mg of sample was homogenized in 5 mL 0.1% trichloroacetic acid and then centrifuged at 10,000 rpm for 15 minutes and finally supernatant was taken. To 1mL of supernatant 3 mL of 5% trichloroacetic acid containing 1% thiobarbituric acid (TBA) was added and heated in a hot water bath for 30 minutes and cooled quickly in cold water bath. It was finally centrifuged at 10,000 rpm for 10 minutes. The absorbance of the supernatant was measured at 530 nm. The concentration of TBARS was measured from its extinction coefficient of 155 μM cm^{-1}. The non-specific turbidity was corrected by subtracting A_{600} from A_{530} value. The TBARS content is finally expressed in n mol g^{-1} dry mass of tissue.

Extraction and estimation of lipoxygenase (EC1.13.11.12)

Lipoxygenase was estimated according to the method of Peterman and Siedow (1985) [47]. Enzyme was extracted by centrifugation at 5000 rpm and re-centrifugation at 17000 rpm in cold using 50 mM sodium-phosphate buffer (p^H-6.5). Then the assay mixture was made containing enzyme extract, 1.3 mM linoleic acid and 1.65 mM sodium-phosphate buffer (p^H-6.5). After incubation of the assay mixture for 1 hour at 25°C, absorbance was taken at 234 nm.

Extraction and estimation of hydroperoxide

Hydroperoxide was estimated by following the method of Devasagayam *et al* (2003) with some necessary modifications [48]. Tissue was extracted with 150 mM tris-HCl (p^H-6.8). The assay mixture contained 0.25 mM H_2SO_4, 250 mM ammonium ferrous sulphate, 100 mM xylenol orange, 4 mM BHT (in 90% methanol) and an aliquot of sample extract. After incubation at room temperature for 30 minutes, triphenyl phosphine (100 mM) was added to the reaction mixture to specify the reduction of hydroperoxide, distinguished from hydrogen peroxide [49]. Then the absorbance was taken at 560 nm.

Extraction and estimation of conjugated diene

The process of Buege and Aust (1978) was followed for the estimation of conjugated diene [50]. Tissue was extracted with chloroform: methanol mixture (2:1) followed by vigorous vortex mixing and then centrifuged for 10 minutes at 2000 rpm. After centrifugation the lower chloroform layer was collected and dried at 45°C under steam of nitrogen. The obtained residue was dissolved in 7 mL of cyclo-hexane and absorbance was taken at 230 nm.

781 ***Extraction and estimation of free carbonyl content***

782 Oxidative damage to proteins was estimated as the content of carbonyl groups following the
783 procedure of Jiang and Zhang (2001) [51]. 500 mg of tissues (seedling) were homogenized
784 with 3 mL of 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM ethylenediamine
785 tetra-acetic acid, 1 mM PMSF (phenyl methyl sulfonyl fluoride), 10 mM dithithreitol and 5
786 μg mL^{-1} leupeptin, 5 μg mL^{-1} aprotinin and 5 μg mL^{-1} antipain (protease inhibitors). The
787 homogenate was centrifuged at 15000×g for 25 min and the supernatant was made free from
788 contaminating nucleic acids by treatment with streptomycin sulfate 50 μg mL^{-1}. An equal
789 volume of 10 mM dinitrophenylhydrazine in 2 M HCl was added to supernatant containing
790 the oxidized protein. These were allowed to stand in the dark at room temperature for 1 hour,
791 with vortex every 10 min. Samples were precipitated with trichloroacetic acid (TCA; 20%
792 final concentration) and centrifuged in a table- top micro centrifuge at 10,000 rpm for 5
793 minutes. The supernatants were discarded and the protein pellets were washed twice more
794 with trichloroacetic acid and then washed three times with 1 mL portions of
795 ethanol/ethylacetate (1:1) to remove any free dinitrophenylhydrazine. The protein samples
796 were re-suspended in 1 ml of 6 M guanidine hydrochloride (dissolved in 20 mM phosphate
797 buffer, pH 2.3) at 37°C for 15 min with vortex mixing. Carbonyl contents were determined
798 from the absorbance at 370 nm using a molar absorption coefficient of 22 mM cm^{-1}.

799 **Germination and early growth phenotypes of indigenous aromatic rice cultivars**
800 **(IARCs) exposed to post-imbibitional dehydration stress (PIDS)**

801 Germination and early growth performances of PIDS-raised IARCs *vis-a-vis* their untreated
802 control were assessed in terms of T_{50} value, relative germination performance (RGP),
803 coefficient of velocity of germination (CVG), germination rate index (GRI), mean

804 germination time (MGT), mean daily germination (MDG), relative growth index (RGI), and
805 vigor index (VI), calculated by the following formulae [52, 53, 54]:

806 T_{50} value: Time (In hour) of 50% germination of seeds sown

807 Relative germination performance (RGP):

808 $$\frac{\text{Percentage of germination under treatment}}{\text{Percentage of germination under control}} \times 100$$

809 Coefficient of velocity of germination (CVG):

810 $$\frac{\sum Ni}{\sum (NiTi)} \times 100$$

811 Germination Rate Index (GRI):

812 $$\sum \left(\frac{Ni}{i}\right)$$

813 Mean Germination Time (MGT):

814 $$\frac{\sum (NiTi)}{\sum Ni}$$

815 Mean Daily Germination (MDG):

816 $$\frac{N}{T}$$

817 Relative growth index (RGI):

818 $$\frac{\text{average dry mass of ten treated seedlings}}{\text{average dry mass of ten control seedlings}} \times 100$$

819 Vigor index (VI):

820 $$\frac{\text{Mean shoot length} + \text{Mean root length}}{\text{Percentage of final germination}}$$

821 ***Determination of relative water content (RWC)***

822 Relative water content was measured by following the method of Barrs and Weatherley
823 (1962) [55]. 10 fresh seedlings of equal length and diameter (4mm) were weighed and floated
824 on distilled water at 25^{o}C for 4 hours. Then the seedlings were blotted and weighed again
825 (Turgid weight). After that, those seedlings were kept for oven dry for 24 hours at 80^{o}C and
826 final weight was taken. The relative water content was calculated by the following formula:

827 $$\text{RWC} = \frac{\text{Fresh weight} - \text{Dry weight}}{\text{Turgid weight} - \text{Dry weight}} \times 100$$

828 **Comparative transcriptomic investigation**

829 Seeds of the experimental IARCs which were screened as drought sensitive (Jamainadu) and
830 drought tolerant (Badshabhog) one, based on quality redox-parameters and physiological
831 phenotyping was imposed with -1.619 MPa PIDS (with PEG-6000) for comparative
832 transcriptome analysis from cDNA libraries prepared from total RNA extracted from the
833 corresponding seedlings. Seven days old PIDS-raised seedlings were collected and planted
834 directly into liquid nitrogen and stored at -80^{o}C for further analysis.

835 ***RNA isolation, cDNA library construction, Illumina sequencing, read mapping,***
836 ***differential gene expression analysis, KEGG pathway analysis and gene ontology (GO)***
837 ***analysis***

838 ***Isolation, Qualitative and quantitative analysis of RNA***

839 Total RNA was isolated from samples using TRIzol reagent according to manufacturer's
840 instruction (Invitrogen, USA). The quality of the isolated RNA was checked on 1%
841 Formaldehyde Denaturing Agarose gel and quantified using Qubit® 2.0 Fluorometer.

Illumina 2 x 150 PE library preparation

The libraries were prepared from samples with input total RNA ~1µg using Illumina TruSeq Stranded mRNA Library Preparation Kit as per the manufacturer's protocol at Xcelris Labs Limited, Ahmedabad, India. Briefly, total RNA was subjected to Oligo dT beads to enrich mRNA fragments, then subjected to purification, fragmentation and priming for cDNA synthesis. The fragmented mRNA was converted into first-strand cDNA, followed by second-strand cDNA synthesis, A-tailing, adapter-index ligation and finally amplified by recommended number of PCR cycles. Library quality and quantity check was performed using Agilent DNA High Sensitivity Assay Kit.

I also tested whether there was any significant effect of dissimilar read sizes in the present analysis by generating MA plot.

Quantity and quality check (QC) of library on Bioanalyzer 2100

The amplified libraries were analyzed on Bioanalyzer 2100 (Agilent Technologies) using High Sensitivity (HS) DNA chip as per manufacturer's instructions.

Cluster Generation and Sequencing

After obtaining the Qubit concentration for the library and the mean peak size from Bioanalyzer profile, library was loaded into Illumina platform for cluster generation and sequencing. Paired-End sequencing allows the template fragments to be sequenced in both the forward and reverse directions. The library molecules bind to complementary adapter oligos on paired-end flow cell. The adapters were designed to allow selective cleavage of the forward strands after re-synthesis of the reverse strand during sequencing. The copied reverse strand was then used to sequence from the opposite end of the fragment.

Bioinformatics analysis (workflow)

Reference guided transcript assembly was performed for all the samples, first by mapping HQ reads on reference genome using hisat2 (v 2.2.1) and then performing transcript assembly by StringTie (v 2.1.4). A consensus set of transcripts was obtained using SringTie merge function which merges together all the gene structures found in any of the samples. Transcript abundance was then estimated using merged transcript consensus again using StringTie and read counts thus obtained for each transcript were taken as input for differential expression analysis using edgeR package. GO and pathway analysis of the differentially expressed transcripts were performed using UniprotKB and KEGG-KAAS server respectively. Overall bioinformatics workflow is graphically represented in figure 17.

[Insert Fig 17 here]

Statistical analysis

Each experiment was carried out twice at different times and had three replicates for each treatment. Results calculated as mean of three replicates ± standard error. Statistical analysis of the data for significance, the "t-test, paired two samples for means" was done using Microsoft Excel 2010, which shows the significant variations between untreated control and different magnitude of post-imbibitional dehydration stress-raised seedlings.

Abbreviations

GO: gene ontology; KEGG: kyoto encyclopedia of genes and genomes; IARC: indigenous aromatic rice cultivar; ABA: abscisic acid; JA: jasmonic acid; SA: salicylic acid; IAA: indole-3-acetic acid; GA: gibberellic acid; PIDS: post-imbibitional dehydration stress; PEG: polyethylene glycol; ROS: reactive oxygen species; TBARS: thiobarbituric acid reactive substances; MPa: megapascal; GB: gigabyte; GTF: gene transfer format; GFF: general feature format; DGE: differential gene expression; DEG: differential expression of genes; KAAS: KEGG automatic annotation server; log2FC: log2 fold change; BS: brassinosteroid; CRSMF: Crop Research and Seed Multiplication Farm; APOX: ascorbate peroxidase; DHAR: dehydroascorbate reductase; GR: glutathione reductase; TAC: total antioxidant capacity; AsA: reduced ascorbate; GSSG: glutathione disulfide; GSH: glutathione; RGP: relative germination performance; CVG: coefficient of velocity of germination; GRI: germination rate index; MGT: mean germination time; MDG: mean daily germination; RGI: relative growth index; VI: vigor index; QC: quality check.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

Seeds of two experimental indigenous aromatic rice cultivars [IARCs *Oryza sativa* L., Cultivars Jamainadu and Badshabhog were collected from Chinsurah Rice Research Station, Chinsurah, Government of West Bengal, India.

Competing interests

Not applicable.

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Authors' contributions

Conceptualization: SB; Methodology: ND; Formal analysis and investigation: ND; Writing - original draft preparation: SB; Writing : ND SB; Funding acquisition: SB, ND; Resources: SB; Supervision: SB. All authors read and approved the final manuscript.

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Table 12: KEGG pathway stats for differentially expressed transcripts

Pathway	***Count***

09100 Metabolism	***5191***
09101 Carbohydrate metabolism	*1244*
09102 Energy metabolism	*480*
09103 Lipid metabolism	*651*
09104 Nucleotide metabolism	*183*
09105 Amino acid metabolism	*783*
09106 Metabolism of other amino acids	*282*
09107 Glycan biosynthesis and metabolism	*234*
09108 Metabolism of cofactors and vitamins	*347*
09109 Metabolism of terpenoids and polyketides	*219*
09110 Biosynthesis of other secondary metabolites	*463*
09111 Xenobiotics biodegradation and metabolism	*305*
09120 Genetic Information Processing	***2396***
09121 Transcription	*378*
09122 Translation	*947*
09123 Folding, sorting and degradation	*733*
09124 Replication and repair	*338*
09130 Environmental Information Processing	***1875***
09131 Membrane transport	*40*
09132 Signal transduction	*1834*
09133 Signaling molecules and interaction	*1*
09140 Cellular Processes	***1837***
09141 Transport and catabolism	*840*
09142 Cell motility	*57*
09143 Cell growth and death	*723*
09144 Cellular community - eukaryotes	*136*
09145 Cellular community - prokaryotes	*81*
09150 Organismal Systems	***824***
09149 Aging	*231*

09158 Development and regeneration	*127*
09159 Environmental adaptation	*466*
09180 Brite Hierarchies	***9882***
09181 Protein families: metabolism	*1674*
09182 Protein families: genetic information processing	*6454*
09183 Protein families: signaling and cellular processes	*1754*
09190 Not Included in Pathway or Brite	***530***
09191 Unclassified: metabolism	*403*
09192 Unclassified: genetic information processing	*15*
09193 Unclassified: signaling and cellular processes	*57*
09194 Poorly characterized	*55*

1088

1089

1090 **Table 13:** Comparative pathway count of uniquely significant up-regulated DEG patheay in

1091 PIDS-raised experimental IARCs Jamainadu (SBND1) and Badshabhog (SBND2)

		Count – SBND1	***Count – SBND2***
09100 Metabolism	09101 Carbohydrate metabolism	70	83
	09102 Energy metabolism	27	19
	09103 Lipid metabolism	34	54
	09104 Nucleotide metabolism	17	13
	09105 Amino acid metabolism	39	49
	09106 Metabolism of other amino acids	17	19
	09107 Glycan biosynthesis and metabolism	12	18
	09108 Metabolism of cofactors and vitamins	18	22

	09109 Metabolism of terpenoids and polyketides	16	20
	09110 Biosynthesis of other secondary metabolites	26	27
	09111 Xenobiotics biodegradation and metabolism	15	5
09120 Genetic Information Processing	09121 Transcription	19	28
	09122 Translation	36	50
	09123 Folding, sorting and degradation	43	47
	09124 Replication and repair	14	26
09130 Environmental Information Processing	09131 Membrane transport	4	3
	09132 Signal transduction	122	90
09140 Cellular Processes	09141 Transport and catabolism	35	75
	09142 Cell motility	3	2
	09143 Cell growth and death	35	36
	09144 Cellular community - eukaryotes	14	2
	09145 Cellular community - prokaryotes	7	3
09150 Organismal Systems	09149 Aging	12	10
	09158 Development and regeneration	15	6
	09159 Environmental adaptation	33	26
09180 Brite Hierarchies	09181 Protein families: metabolism	106	108
	09182 Protein families: genetic information processing	352	450
	09183 Protein families: signaling and cellular processes	91	115
09190 Not Included in Pathway or Brite	09191 Unclassified: metabolism	18	24
	09192 Unclassified: genetic information processing	0	1

	09193 Unclassified: signaling and cellular processes	4	1
	09194 Poorly characterized	6	4
Total result		**1260**	**1436**

Figure legends

Fig1: Comparative representation of the status of Halliwell-Asada pathway in post-imbibitional dehydration stress (PIDS)-raised IARCs, Jamainadu (A) and Badshabhog (B) *vis-a-vis* their untreated control (Unt. Cont.).

Fig2: *In situ* localization of hydrogen peroxide in roots observed through laser confocal microscopy (A & B) and visualization of hydrogen peroxide through TMB staining (C & D) and superoxide through NBT staining (E & F) in post-imbibitional dehydration stress [PIDS (PEG-6000 induced)]–raised experimental IARCs (*Oryza sativa* L. Jamainadu and Badshabhog) as compared to their respective untreated control.

Fig3: Comparative representation of the status of sensitive redox biomarkers of post-imbibitional dehydration stress (PIDS)-raised IARCs, Jamainadu (A) and Badshabhog (B) *vis-a-vis* their untreated control (Unt. Cont.).

Fig4: Germination and early growth phenotypes of IARCs, Jamainadu and Badshabhog exposed to post-imbibitional dehydration stress [PIDS (PEG-6000 induced)] *vis-a-vis* their untreated control (Unt. Cont.). Results are mean of three replicates ± standard error. *Significant from control at 0.05 level (t-test). **Significant from control at 0.01 level (t-test).

Fig 5: QC of total RNA on 1% Formaldehyde Agarose gel [SBND1: PIDS (-1.619 MPa)-raised seedlings of IARC, Jamainadu, SBND2: PIDS (-1.619 MPa)-raised seedlings of IARC, Badshabhog]

Fig 6: Library profile of PIDS (-1.619 MPa)-raised seedlings of IARC, Jamainadu on Agilent DNA HS Chip

Fig 7: Library profile of PIDS (-1.619 MPa)-raised seedlings of IARC, Badshabhog on Agilent DNA HS Chip

Fig 8: Different class codes and their descriptions (https://ccb.jhu.edu/software/stringtie/gffcompare.shtml)

Fig 9: Venn diagram showing the number of genes expressed in both the PIDS-raised seedlings of experimental IARCs [SBND1: PIDS (-1.619 MPa)-raised seedlings of IARC, Jamainadu, SBND2: PIDS (-1.619 MPa)-raised seedlings of IARC, Badshabhog]

Fig 10: Heatmap representing most significant genes expressed in all four samples was plotted using log10 of normalized read count values (CPM) for SBND1-Vs- SBND2, where shades of blue represents downregulated genes and shades of red represents highly expressed genes. [SBND1: PIDS (-1.619 MPa)-raised seedlings of IARC, Jamainadu, SBND2: PIDS (-1.619 MPa)-raised seedlings of IARC, Badshabhog]

Fig 11: MA plot showing differentially expressed transcripts in PIDS (-1.619 MPa)-raised seedlings of IARCs, Jamainadu Vs Badshabhog combination. On X-axis normalized counts for all the samples and on Y-axis log2foldchange are plotted. Points colored are with red if the adjusted p value/q-value is less than 0.05 and black if the adjusted p value/q-value is greater than 0.05

Fig 12: Volcano plots of the distribution of expressed transcripts in PIDS (-1.619 MPa)-raised seedlings of IARCs, Jamainadu Vs Badshabhog combination. Red corresponds to transcripts with adjusted p value/q-value < 0.05

Fig 13: GO distribution for SBND1-Vs-SBND2 differentially expressed transcripts [SBND1: PIDS (-1.619 MPa)-raised seedlings of IARC, Jamainadu; SBND2: PIDS (-1.619 MPa)-raised seedlings of IARC Badshabhog]

Fig 14: GO of significant gene number difference under all significant DGE-GO (A) and GO-WEGO plot showing comparative significant DGE-GO (B) in both the experimental IARCs (Jamainadu and Badshabhog).

Fig 15: GO of significant gene number difference under uniquely up-regulated (significant_DGE_GO) (A) and GO-WEGO plot showing comparative nature of uniquely up-regulated significant DGE-GO (B) in both the experimental IARCs (Jamainadu and Badshabhog).

FIG 16: Plant hormone signal transduction KEGG pathway: Purple color entry has been assigned to at least one gene entry whereas a pink color entry corresponds to an ortholog table entry, which corresponds to a gene that is a part of a functional unit on the pathway.

FIG 17: Graphical representation of overall bioinformatics workflow.

Figures

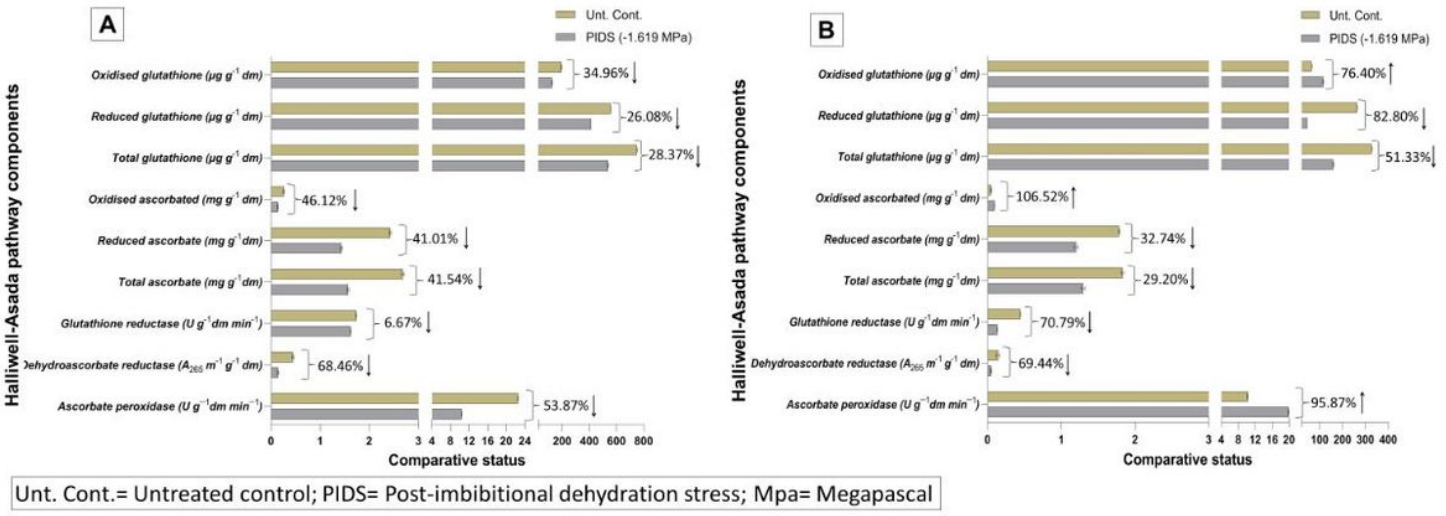


Figure 1

"See the Supplemental Files section for the complete figure caption".

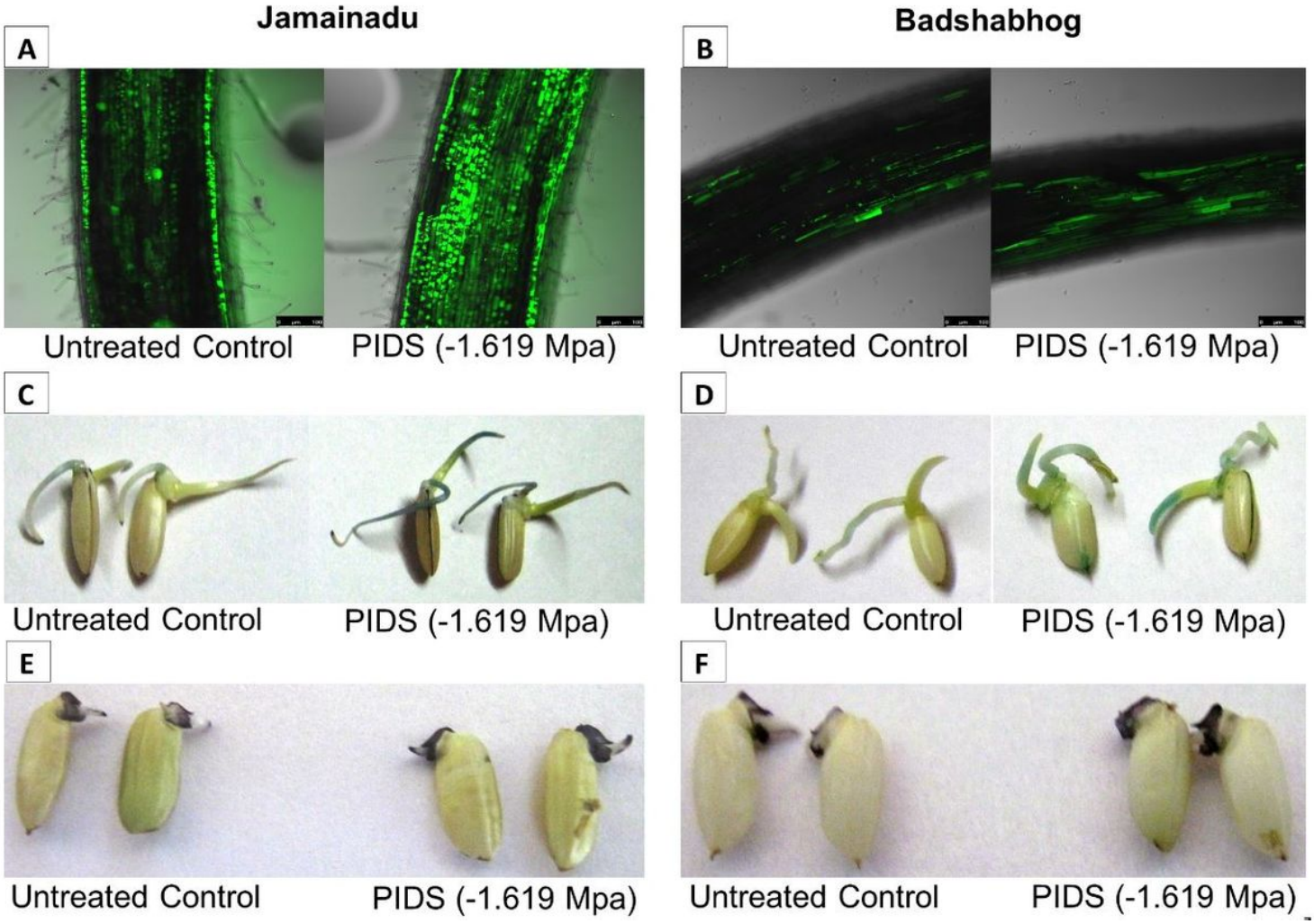
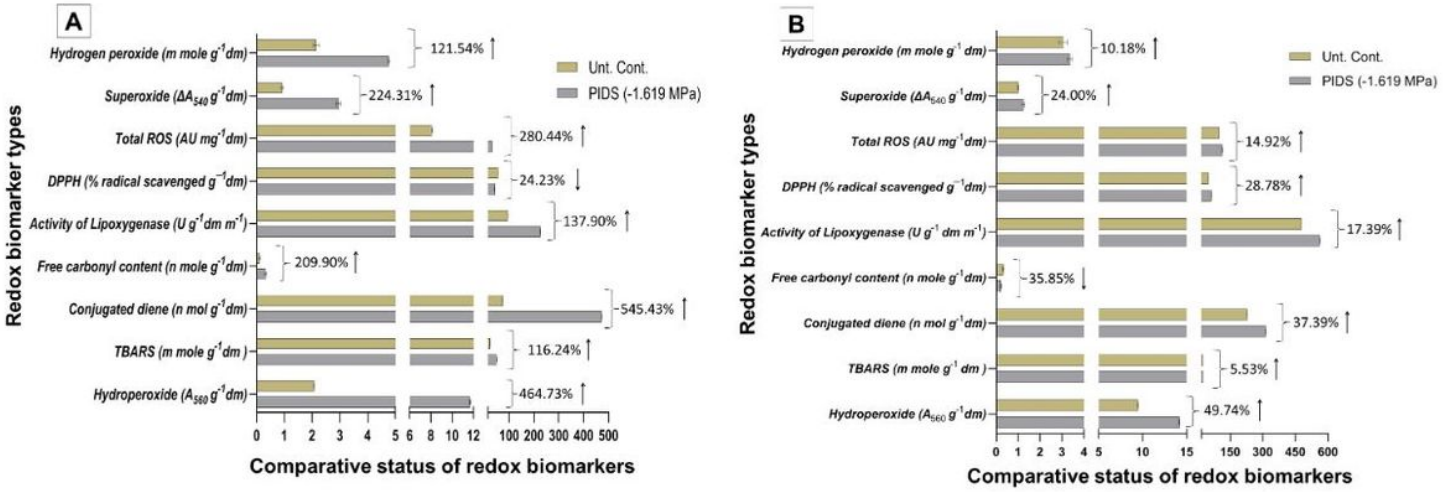


Figure 2

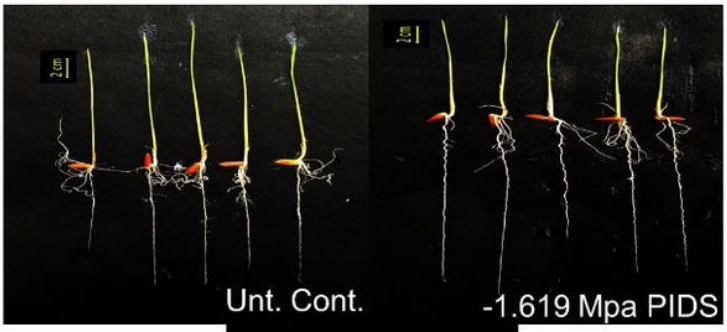
"See the Supplemental Files section for the complete figure caption".

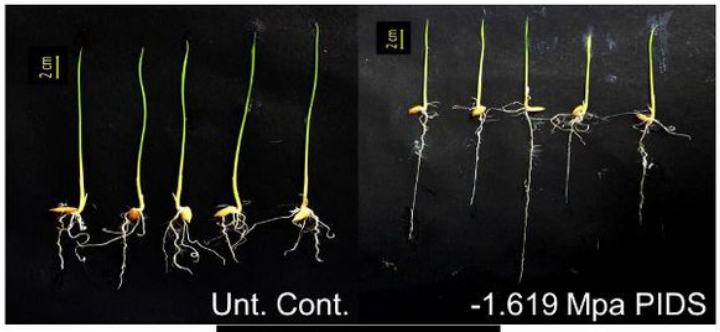


Unt. Cont.= Untreated control; PIDS= Post-imbibitional dehydration stress; Mpa= Megapascal

Figure 3

"See the Supplemental Files section for the complete figure caption".





Early growth performances	Land races of Aromatic rice	Post-imbibitional Dehydration Stress [PIDS]	
		Untreated control	-1.619 MPa
T_{50} (hour)	Jamainadu	26.09±0.05	150±0.54
	Badshabhog	25±0.23	30±0.27
Vigor Index (VI)	Jamainadu	0.148±0.001	0.067±0.001**
	Badshabhog	0.122±0.016	0.138±0.005
Relative Germination Performance (RGP)	Jamainadu	100.00±0	51.11±0.25**
	Badshabhog	100.00±0	92.00±0.22**
Relative Growth Index (RGI)	Jamainadu	100.00±0	95.65±0.24*
	Badshabhog	100.00±0	109.28±0.98
Relative Water Content (RWC)	Jamainadu	91.7±0.22	83.50±0.17**
	Badshabhog	87.05±0.20	82.44±0.14**
Co-efficient of velocity of germination (CVG)	Jamainadu	23.35±0.06	18.96±0.08**
	Badshabhog	22.39±0.05	22.10±0.04*
Germination Rate Index (seed day^{-1})	Jamainadu	90.76±0.08	36.22±0.10**
	Badshabhog	43.15±0.07	36.74±0.05**
Mean Germination Time (day)	Jamainadu	4.28±0.06	4.55±0.06**
	Badshabhog	4.47±0.06	4.53±0.05
Mean Daily Germination (day^{-1})	Jamainadu	7.5±0.05	3.83±0.06**
	Badshabhog	6.25±0.05	4.83±0.04**

Figure 4

"See the Supplemental Files section for the complete figure caption".

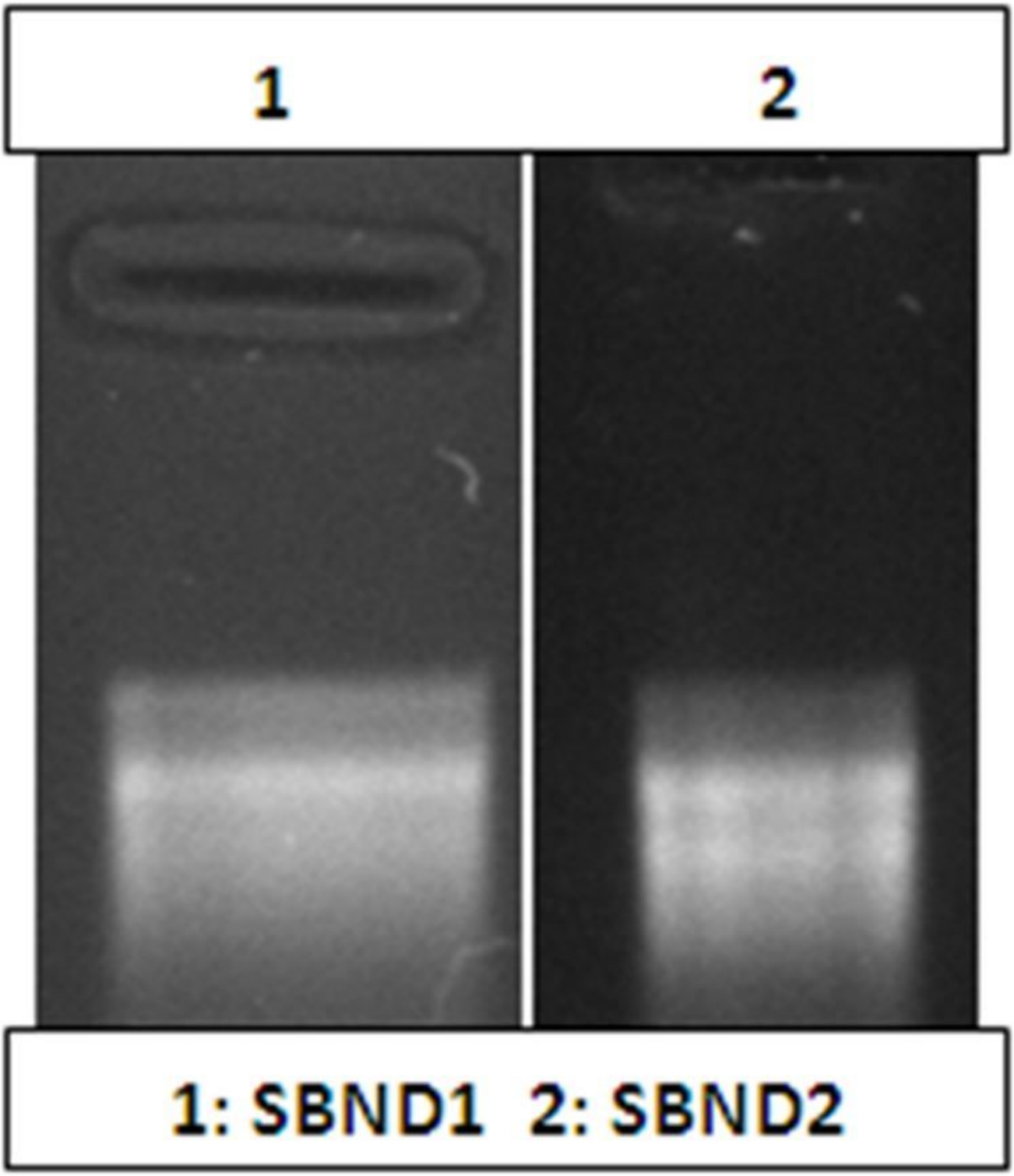
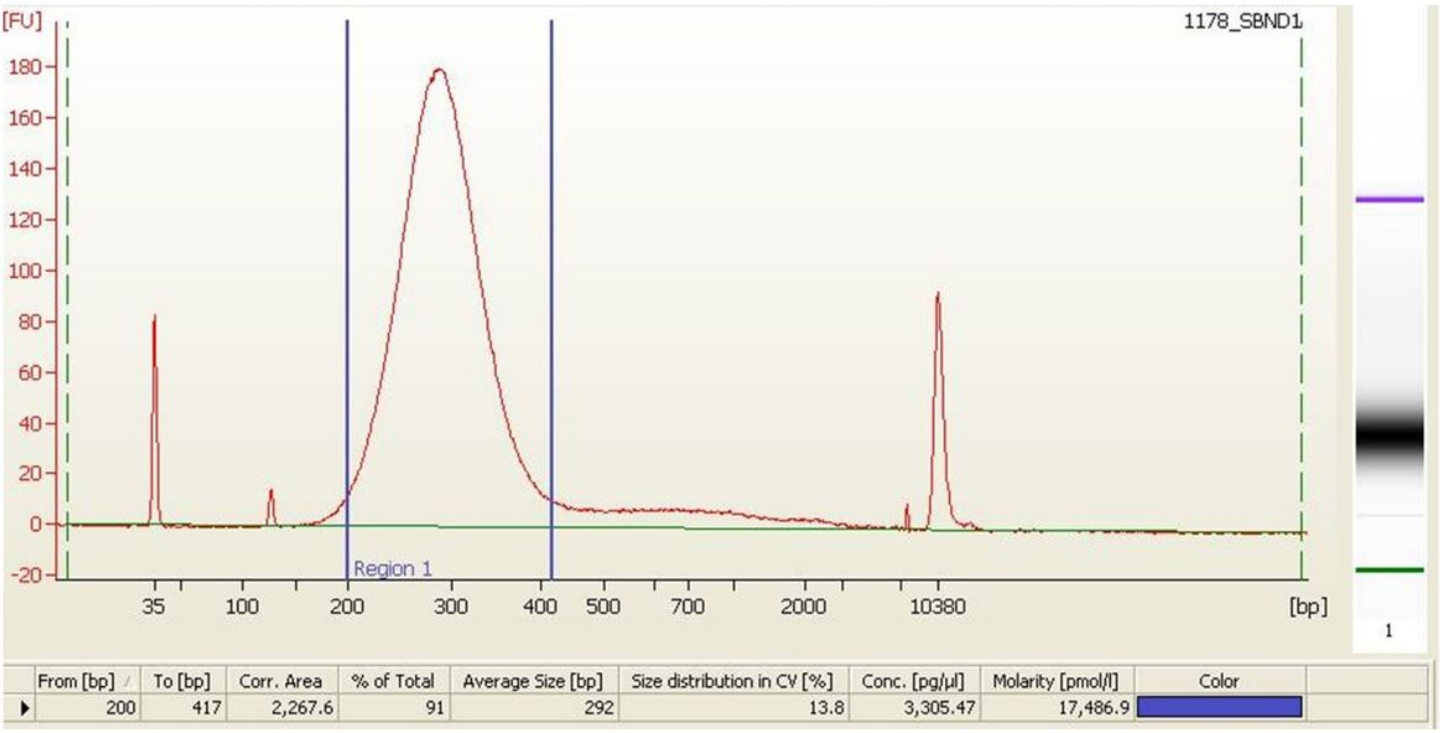


Figure 5

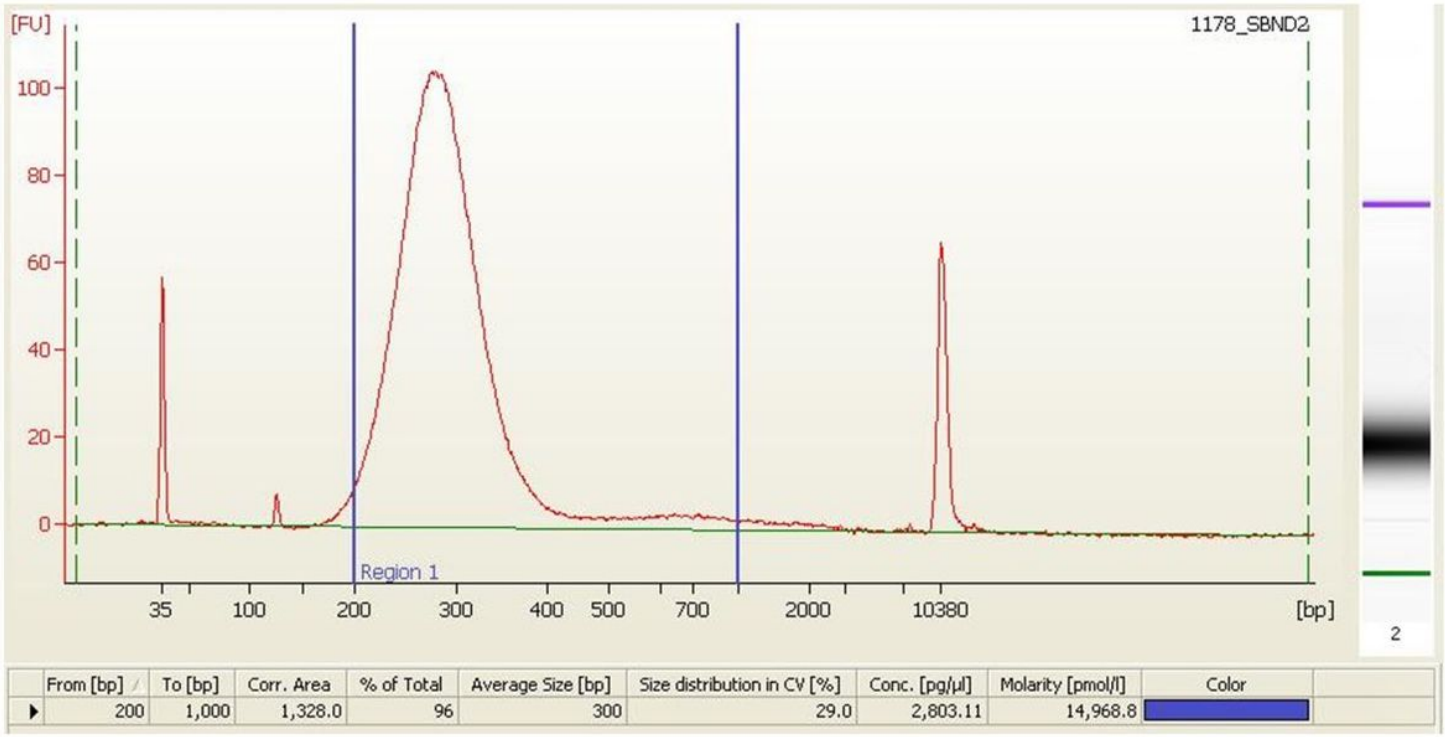
"See the Supplemental Files section for the complete figure caption".



From [bp]	To [bp]	Corr. Area	% of Total	Average Size [bp]	Size distribution in CV [%]	Conc. [pg/µl]	Molarity [pmol/l]	Color
200	417	2,267.6	91	292	13.8	3,305.47	17,486.9	

Figure 6

"See the Supplemental Files section for the complete figure caption".



From [bp]	To [bp]	Corr. Area	% of Total	Average Size [bp]	Size distribution in CV [%]	Conc. [pg/µl]	Molarity [pmol/l]	Color
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Figure 7

"See the Supplemental Files section for the complete figure caption".

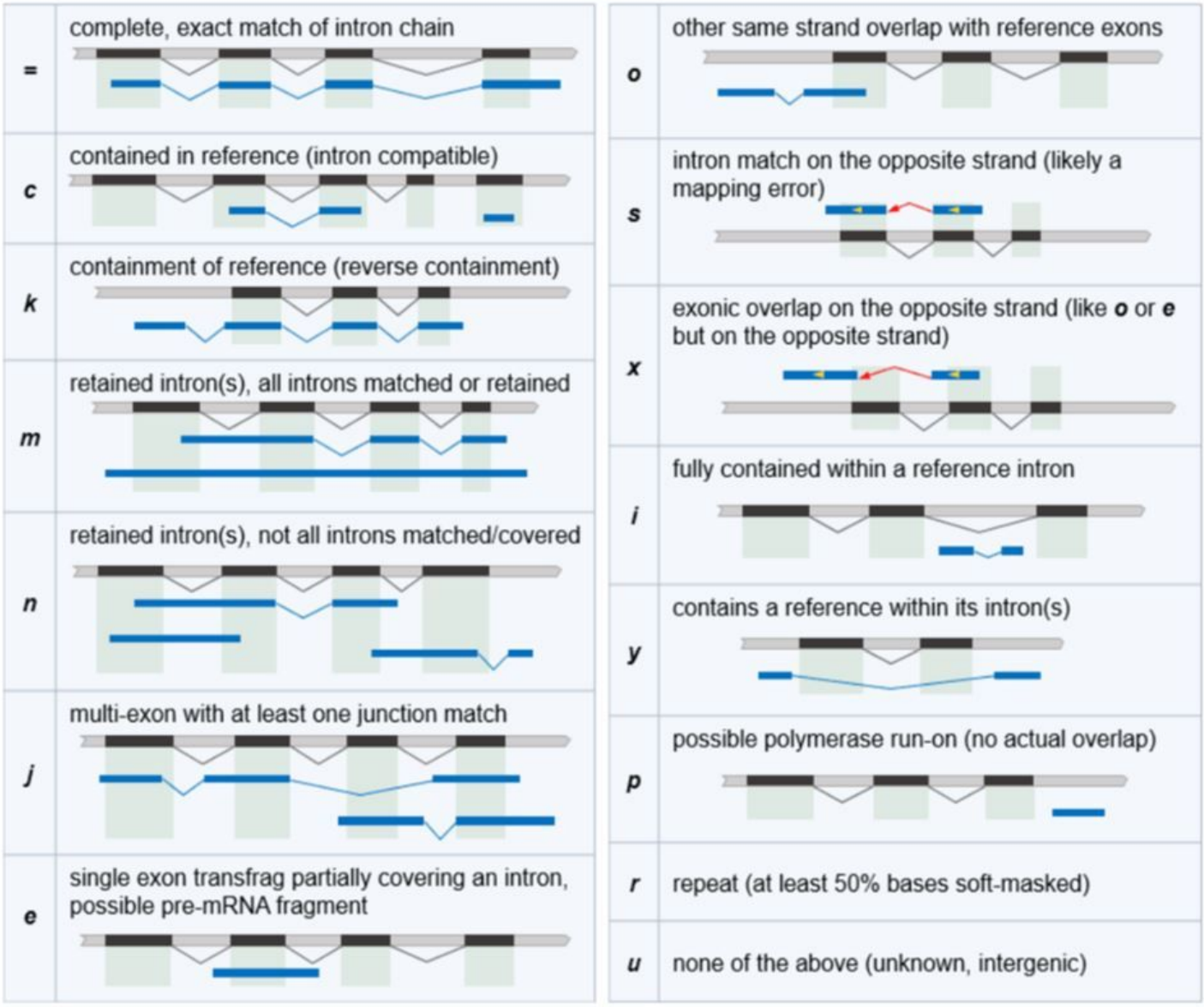

Figure 8

"See the Supplemental Files section for the complete figure caption".

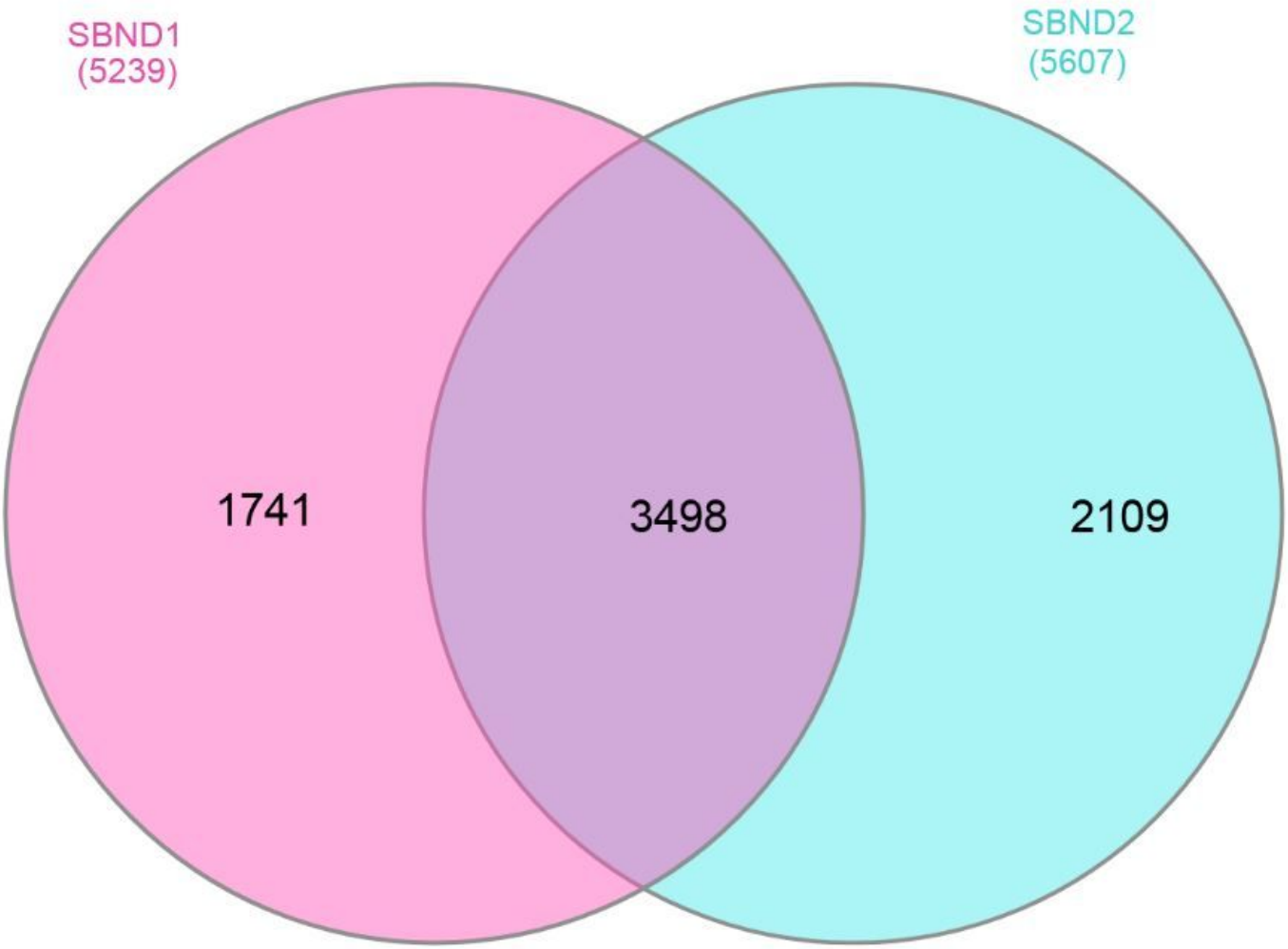

Figure 9

"See the Supplemental Files section for the complete figure caption".

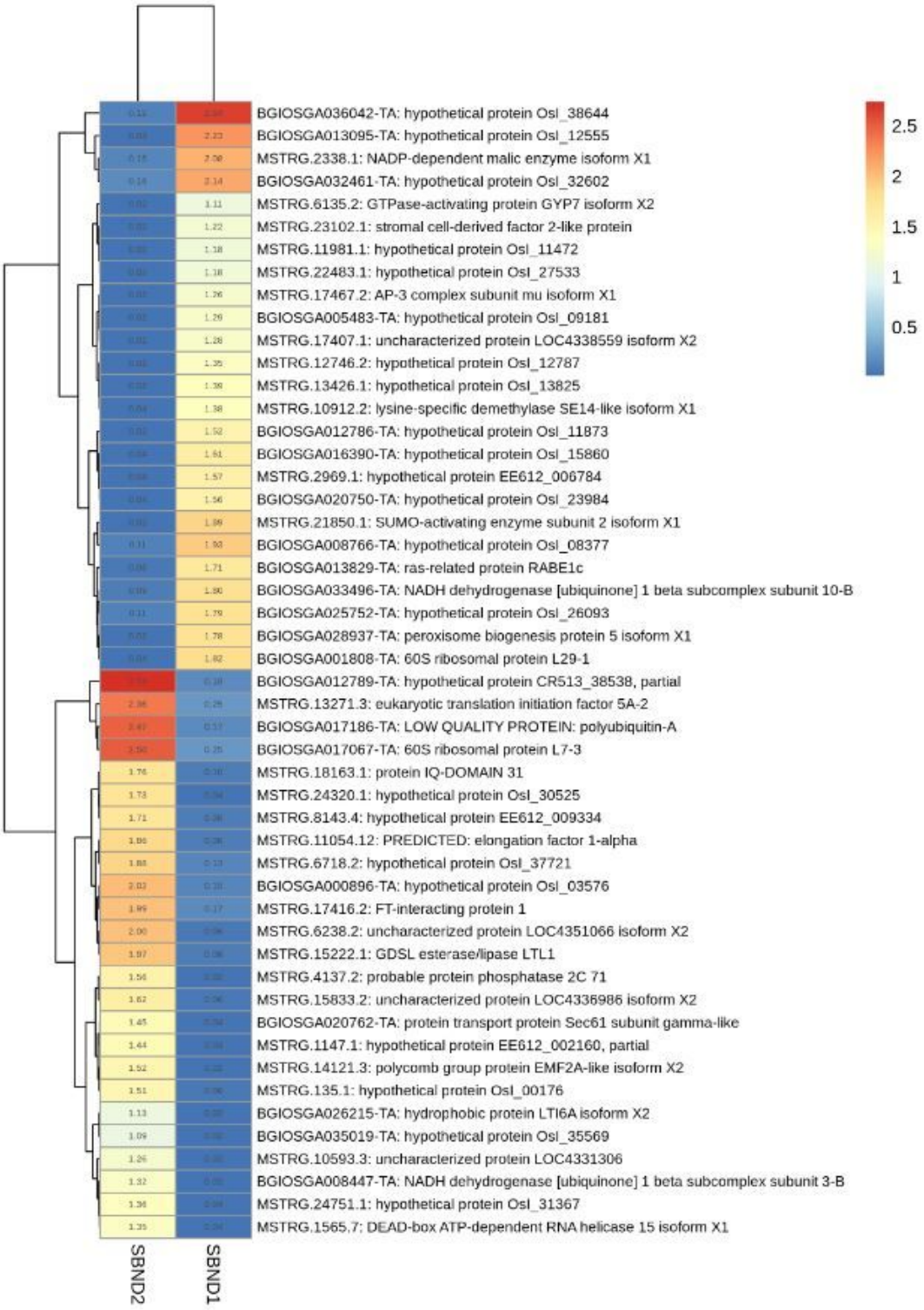

Figure 10

"See the Supplemental Files section for the complete figure caption".

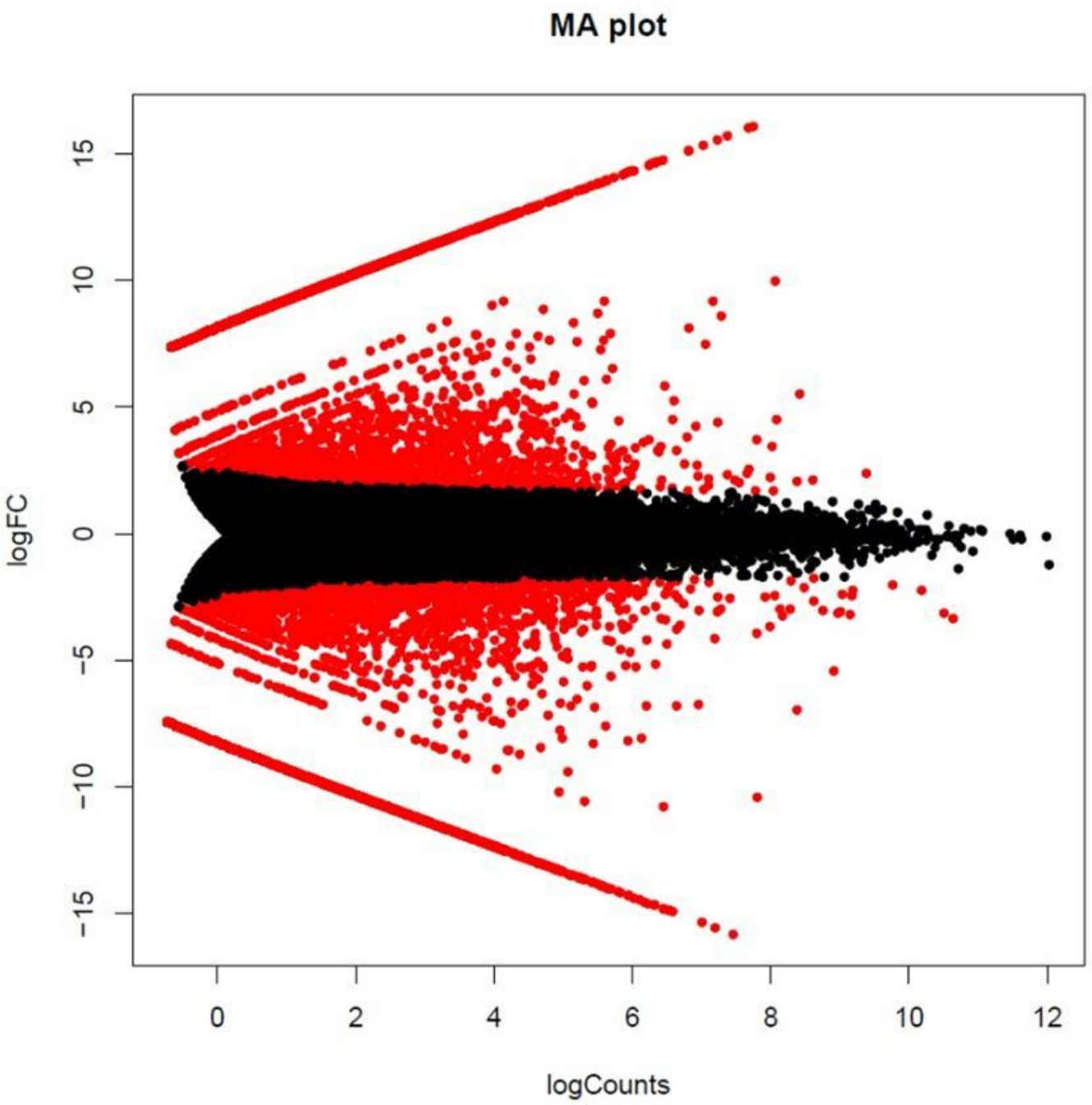

Figure 11

"See the Supplemental Files section for the complete figure caption".

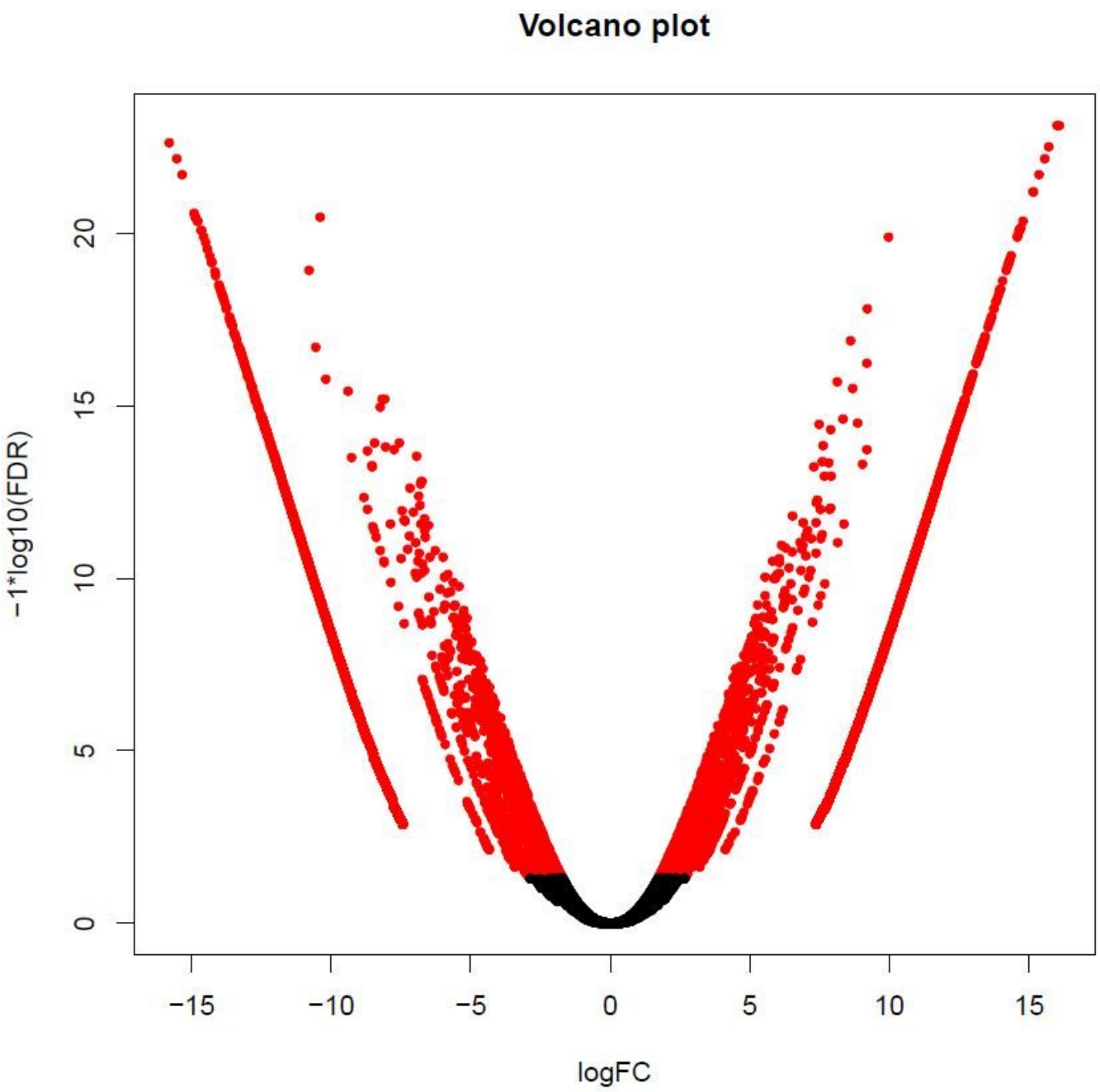

Figure 12

"See the Supplemental Files section for the complete figure caption".

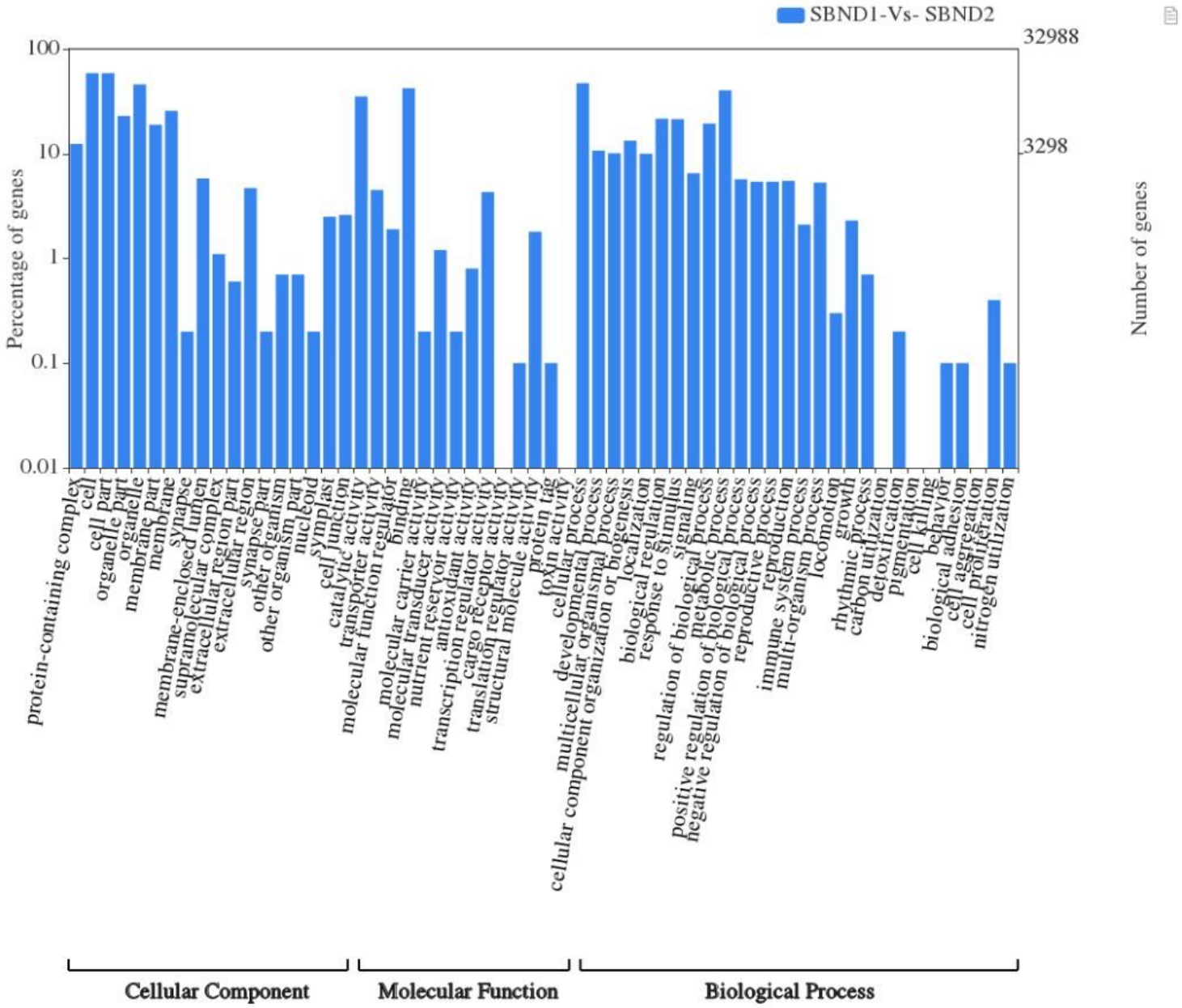

Figure 13

"See the Supplemental Files section for the complete figure caption".

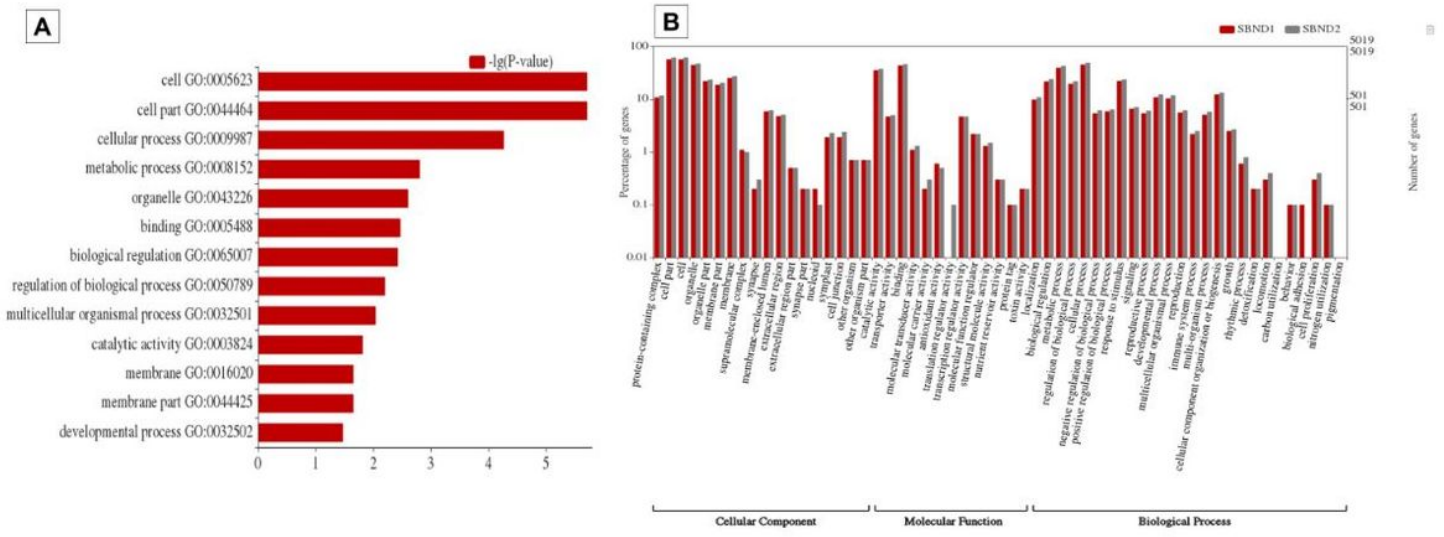

Figure 14

"See the Supplemental Files section for the complete figure caption".

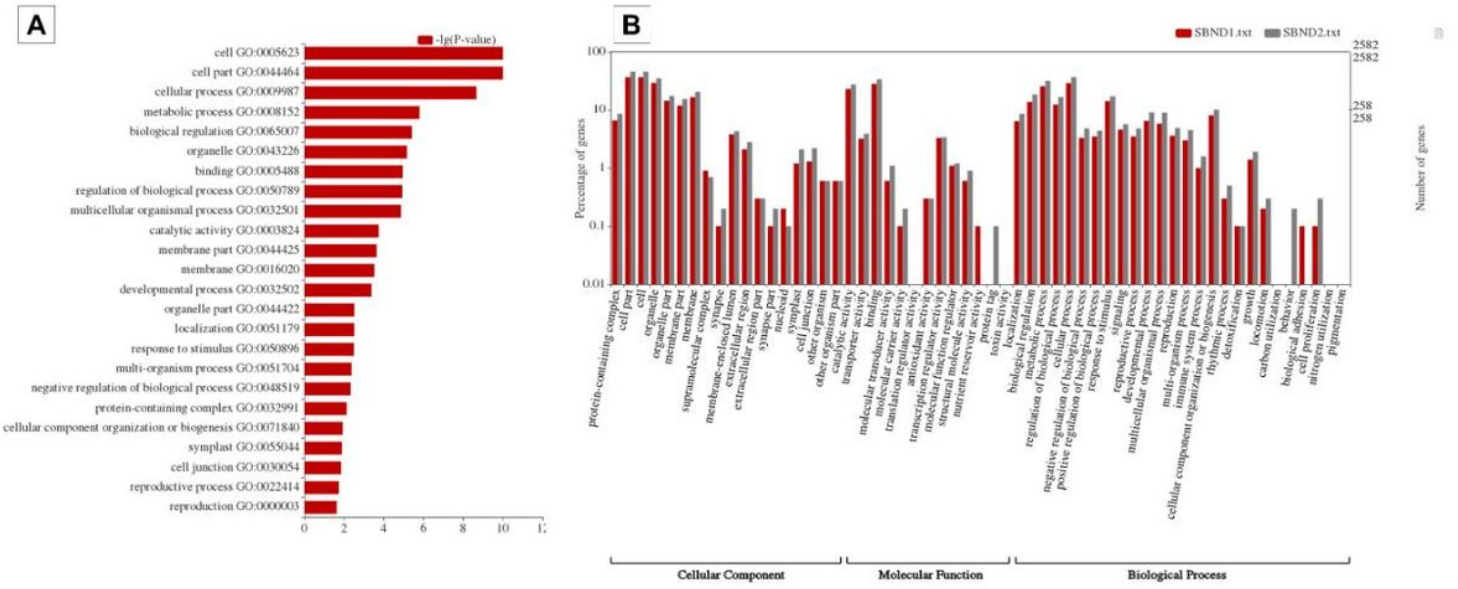

Figure 15

"See the Supplemental Files section for the complete figure caption".

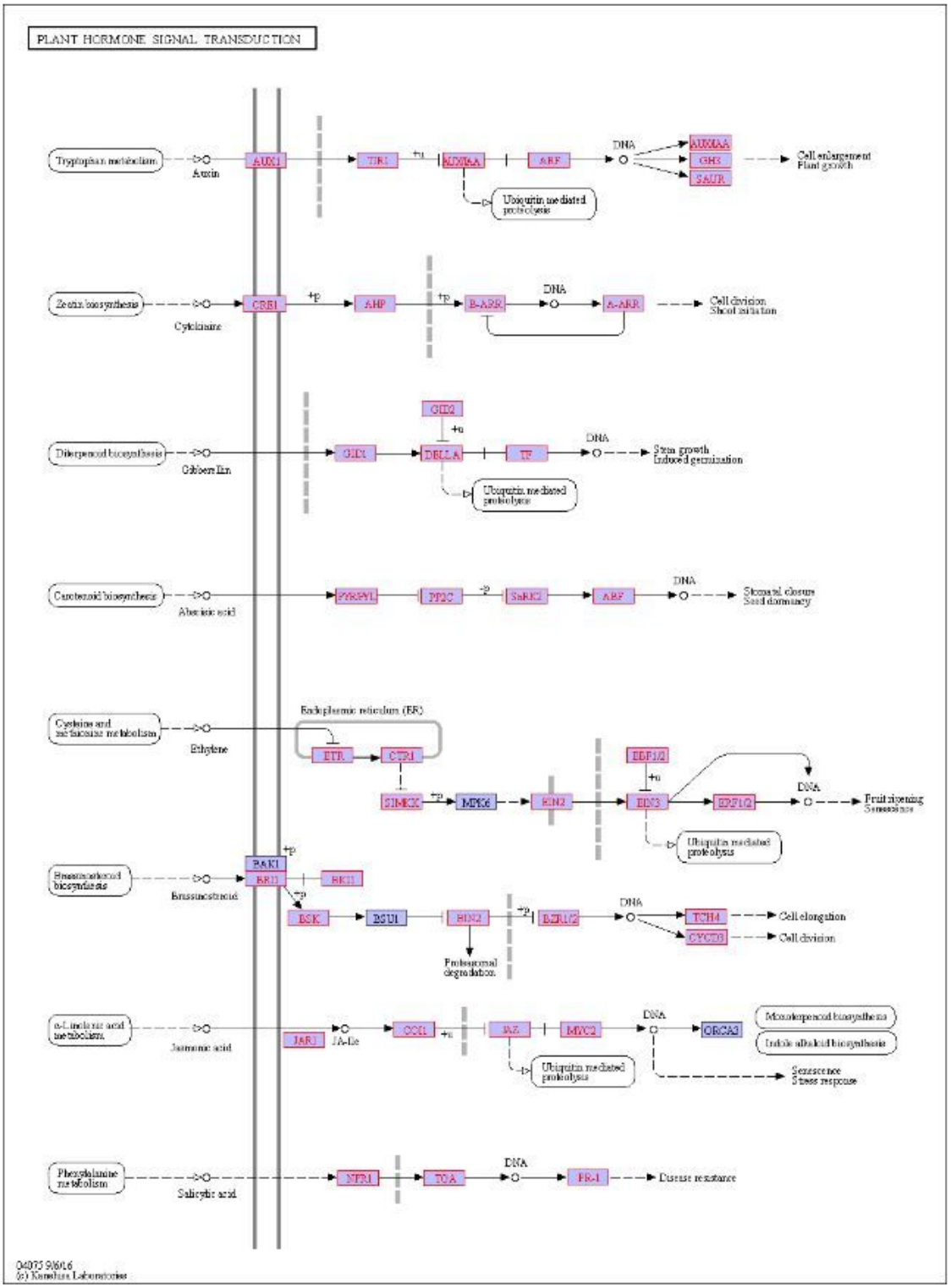

Figure 16

"See the Supplemental Files section for the complete figure caption".

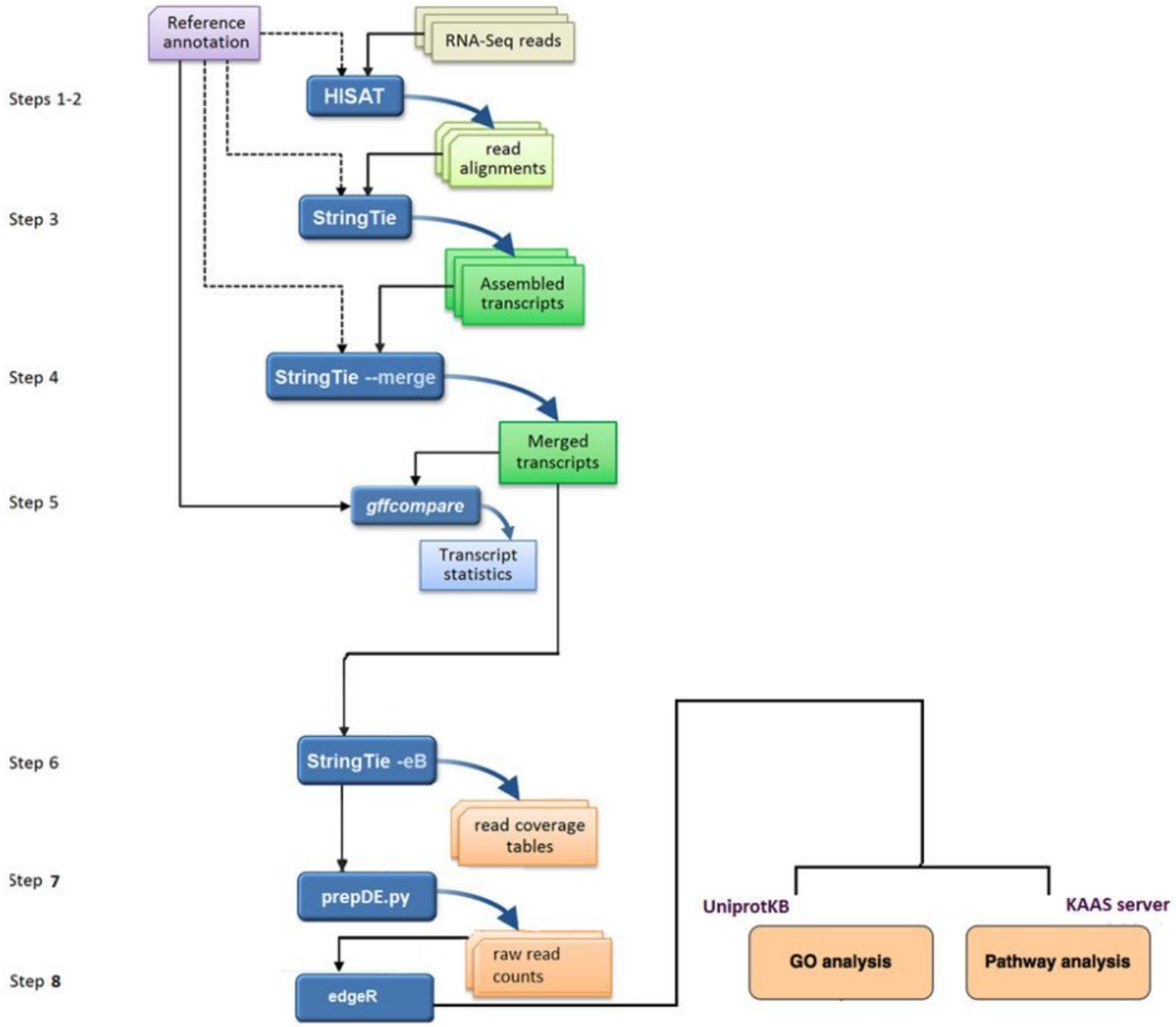

Figure 17

"See the Supplemental Files section for the complete figure caption".

Supplementary Files

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- FigureCaptions.pdf