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Research Article

Transcriptome Analysis of Chilling-responsive Gene Expression Profiles in Sugarcane (*Saccharum officinarum* L.) with Different Response to Nano-graphene

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Abstract:

Sugarcane (*Saccharum officinarum* L.) is very important crop for sugar and energy production worldwide with highly economic-value, growing in tropical and subtropical regions. However, abiotic stresses including chilly adversely affect its growth and productivity. With growing the consideration of nanotechnology in reduction the adverse effects of environmental stresses in plants, the present study investigates the transcriptome responses of graphene nanoparticles (GrNPs) in sugarcane (Guitang 49 cultivar) under chilly stress. The treatments were set as control, chilling (4ºC), GrNPs [50 part per million (ppm)] and chilling + GrNPs. Samples were collected and subjected to physiological determination and transcriptome analyses. Chilly treatment induced an increase in rate of membrane leakage and malondialdehyde (MDA) content. The combined applications of Chillying + GrNPs ameliorated the oxidative damage. RNA-Seq-based transcriptome analysis followed by the Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis pointed out that the mainly enriched KEGG pathways that were detected in almost all treated combinations are: plant hormones signal transduction, phenylpropanoid biosynthesis, starch and sucrose metabolism, carotenoid biosynthesis and photosynthesis antenna proteins. The detected up and downregulated DEGS involved in these pathways could be useful to understand the mechanisms underlying the response to graphene.

1. Introduction

Sugarcane (*Saccharum officinarum* L.) is very important crop for sugar and energy production worldwide (Li and Yang, 2015). It is cultivated in several tropical and subtropical countries where it is considered as a major cash and industrial crop (Li et al., 2015; Mehareb et al., 2022). It stores a great amount of sugar in its stem as other members of Poaceae family (Figueroa-Rodriguez et al., 2019).

The sugarcane crop typically grows for eight to nine months (REF). The optimum temperature for better growth and development of sugarcane is ranged from 25°C to 30°C (Elsheery et al., 2020; Dharshini et al., 2016). Chilly stress causes severe damage, less growth and development to sugarcane plants (Li et al., 2015). Cell membrane property, osmotic potential, ice crystal development as well as concentration and distribution of reactive oxygen species (ROS) are altered due to chilly stress (Dong et al., 2009). The extreme production of ROS under chilly stress resulted in cellular destruction, degradation of large molecules included DNA, RNA and proteins as well as photosynthetic inhibition (Li et al., 2019; Sun et al., 2009; Zhang et al., 2021). Stimulation of numerous physiological and molecular mechanisms is required in plants for developing various strategies to reduce adverse effects of low temperature and enhance cold-tolerance (Huang et al., 2010; Haghighi et al., 2014; Elsheery et al., 2020). Exploration of changes in the expression of genes as well as new strategies involved in adaptation to chilling stress became the main interest of various researchers to recognize the mechanisms of chilly tolerance and employ it for varieties improvement and functional divergence in sugarcane such as yield and sugar content specially under obvious environmental conditions (Yang et al., 2017; Su et al., 2020; Rehman et al., 2020; Wang et al., 2020; Huang et al., 2022).

Biological application of nanoparticles (NPs) is one of important low cost strategies to improve plant tolerance against different stresses (Haghighi et al., 2014; Ashkavand et al., 2015; Zulfiqar and Ashraf, 2021). For their unique properties and applications (Khan et al., 2023; Zhang et al., 2019), NPs has been found to have great prospective usages in agriculture (Azimi et al., 2014). Previous studies reported that NPs could function to alter plant genome and to enrich the biosynthesis of bioactive compounds required for protecting the plant against different stresses (Hatami et al., 2016; Kim et al., 2012). The promising potential of NPs for mitigating chilly stress (Elsheery et al., 2008; Huang et al., 2010) could be explored further.

Graphene nanoparticles (GrNPs) are one of Carbon-based nanomaterials that were used in several scientific fields, such as drug delivery, biosensors, tissue engineering plant improvement (Kitko and Zhang, 2019; Verma et al., 2019; Park et al., 2020). Recently, numerous studies have focused on the physiological and/or phenotypic response to graphene applications (Chen et al., 2021; Elsheery et al., 2020; Park et al., 2020), while molecular

response to graphene is rarely documented. Also, studies on the interactive effects of GrNPs under chilly stress on plants and their molecular mechanisms related to their capability to alleviate chilly stress in plants is unrevealed yet. The present study aimed to elucidate the transcriptome information of various differential expressed genes among different metabolic pathways in response to chilly stress. Also to illustrate the molecular response to foliar application of GrNPs on stressed plants, willing to provide new understanding of amelioration capability of GrNPs for chilling tolerance in sugarcane plants.

2. Materials and Methods

2.1. Plant materials and growth conditions

This study was conducted using moderately chilling tolerant cultivar of sugarcane (Guitang 49) that developed by Guangxi Sugarcane Research Institute, Guangxi Academy of Agricultural Sciences, China as a plant material. Bud node was submerged with flowing water for 24 h to stimulate buds develop then cultured in pots filled with sand soil in the growth chamber at 28 °C. The seedlings were allowed to grow to the late tillering phase (75 days). Seedlings were watered, fertilized according to common cultivation practices for sugarcane plants. Nutritional element were added in rates of 70, 50 and 100 mg/kg soil from Nitrogen, Potassium and Phosphorous respectively. Also, Hoagland solution (150 mL/pot) was applied once in two weeks. Chilly treatment was carried out at 4°C for six days. Culture temperature control plants kept at 28 °C. Plant samples were collected after the end of chilly treatment and were packed with dry ice then stored at -80°C for further determinations.

2.2. Foliar application of nano Graphene

Graphene nanoribbons (GrNPs); 2–15 μm × 40–250 nm, alkyl functionalized, PCode: 1002232815) (Sigma Aldrich, USA) were used as foliar spray (50 ppm) (Zhang et al., 2015) on the leaves of planted cultivar (Guitang 49) seven days before the chilly treatment. The GrNPs were dispersed in distilled water for 3 h of ultrasonication. Seedlings sprayed with distilled water were considered as control for GrNPs treatments.

2.3. Photosynthesis pigments

Photosynthesis pigments including: Chlorophyll a (Chl a) and b (Chl b) and carotenoids (CAR), were extracted and determined following Welburn methods (Welburn, 1994).

2.4. Lipid peroxidation and electrolyte leakage

Lipid peroxidation and membrane leakage were detected to evaluate the membrane stability under all experimental conditions. Malondialdehyde (MDA) content as the equated product of lipid peroxidation was determined in fresh leaf slices (0.5 to 1.0 g) according to Heath and Packerk's method (Heath and Packer, 1968). Rate of electrolyte leakage from the membrane of fresh sugarcane leaves was utilized to evaluate membrane permeability. Electrolyte leakage was expressed as percentage of total conductivity according to Omar et al., (Omar et al., 2012).

2.5. Transcriptome analysis

2.5.1. RNA extraction and purity determination

Sugarcane leaves were collected and frozen immediately at -80 ºC. Total RNA was extracted from sugarcane leaves using Trizol (TriQuick Reagent-R1100, Beijing, China). Agaros gel (1%) was prepared for monitoring RNA degradation and contamination. RNA purity was checked using the NanoPhotometer® spectrophotometer (IMPLEN, CA, USA). Qubit® RNA Assay Kit in Qubit® 2.0 Flurometer (Life Technologies, CA, USA) was used for detecting RNA concentration.

2.5.2. Library preparation for Transcriptome sequencing

1.5 μg RNA was used as input material for each sample. NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA) was used for producing the sequencing libraries following manufacturer's recommendations. cDNA fragments were selected preferentially of 250~300 bp in length and filtered with AMPure XP system (Beckman Coulter, Beverly, USA). PCR products were purified (AMPure XP system) and library quality was assessed on the Agilent Bioanalyzer 2100 system.

2.5.3. Differential expression analysis

Differential expression analysis of two conditions/groups was achieved using the DESeq R package (1.10.1). Benjamini and Hochberg's approach was used to correct the resulting *P* values and control the false discovery rate. Genes with corrected *P*-value <0.05 found by DESeq were assigned as differentially expressed. The threshold for significantly differential expression was set as Qvalue < 0.005 & log2 (foldchange) >1.

2.5.4. Quantification of gene expression levels

Transcription levels were assessed by RSEM (Li and Dewey, 2011) for each sample. The gene expression was normalized using the value of reads per kilobase per million reads (RPKM). Read count was obtained after mapping back the clean data onto the assembled transcriptome.

2.5.5. Gene functional annotation

Seven databases were used for homology-based annotation of gene function: Nt (NCBI non-redundant nucleotide sequences), Pfam (Protein family), Nr (NCBI non-redundant protein sequences), Swiss-Prot (A manually annotated and reviewed protein sequence database), KOG/COG (Clusters of Orthologous Groups of proteins), KO (KEGG Ortholog database) and GO (Gene Ontology).

2.5.6. Gene Ontology (GO) enrichment analysis

GOseq R packages adjusted for gene length bias in DEGs based on Wallenius non-central hyper-geometric distribution (Young et al., 2010) were used for performing gene ontology (GO) enrichment analysis of the differentially expressed genes (DEGs).

2.5.7. KEGG pathway enrichment analysis

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database (Kanehisa et al., 2008) was used for

illustrating high-level functions and values of the biological system. Statistical enrichment of differential expression genes in KEGG pathways was performed using KOBAS software (Mao et al., 2005).

3. Results and discussion

3.1. Photosynthesis pigments

Determination of photosynthesis pigments showed that chilly treatment induced a significant reduction in all chlorophyll a (Chl a) and chlorophyll b (Chl b) while carotenoids (CAR) were increased. On another hand GrNPs treatment showed a positive correlation with photosynthesis pigments contents (Figure 1: A, B, C). GrNPs treatment induced a significant increase in Chl a, Chl b and CAR in both stressed and non-stressed plants (Figure 1: A, B, C) comparing with chilly treated and control plants, respectively. Thus, indicate to the improvement in photosynthesis pigments content with GrNPs treatment. It was reported that treatment with several types of NPs induced a significant improvement in photosynthesis process in various species (Shi et al.,2013; Siddique et al., 2014; Djanaguiraman et al., 2018). Positive effect of GrNPs treatment on plant growth was reported in garlic (Chakravarty et al., 2015). In sugarcane higher carotenoid contents post GrNPs application induced photoprotection subsequently, improved photosynthesis process (Elsheery et al., 2020).

3.2. Lipid peroxidation and electrolyte leakage

Determination of MDA as a product of lipid peroxidation as well as rate of membrane leakage were used as a powerful indicator for changes in membrane stability under different stresses of all experimental conditions. Obtained results displayed that chilling stress induced a significant increase in MDA content as well as leakage rate comparing with the control. While GrNPs treatment caused a significant reduction in MDA and leakage rate of electrolyte in both stressed and non-stressed plants (Figure 2 A, B). Thus, pointed to the oxidative stress of chilling treatment due to the acute production of ROS (Zhu et al., 2013) and suggesting that GrNPs can mitigate oxidative stress. It has been found a direct enhancement in plant defense enzymes, hormone content, and the expression of stress-related genes with treatment with GrNPs in soybeans and *Zea mays* (Lopes et al., 2022; Yazicilar et al., 2024).

3.3. Summary of transcriptome data

Tested cultivar of sugar cane responded differently under experimental treatments which promote the evaluation of differential changes in gene expression underlying different responses. RNA-Seq-Based transcriptome analysis of 12 samples were generated after removing adaptors and low-quality reads, on average, 52358547 (control), 49369533 (chilly stress), 56566801 (Gr) and 45575989 (Gr + Ch). As minimum 94.22 % of the clean reads presented eligible Q30 (%) values in all libraries. The minimum GC ratio of clean reads was higher than 53% (Table 1), representative that the sequencing data were of adequately high quality for subsequent analyses.

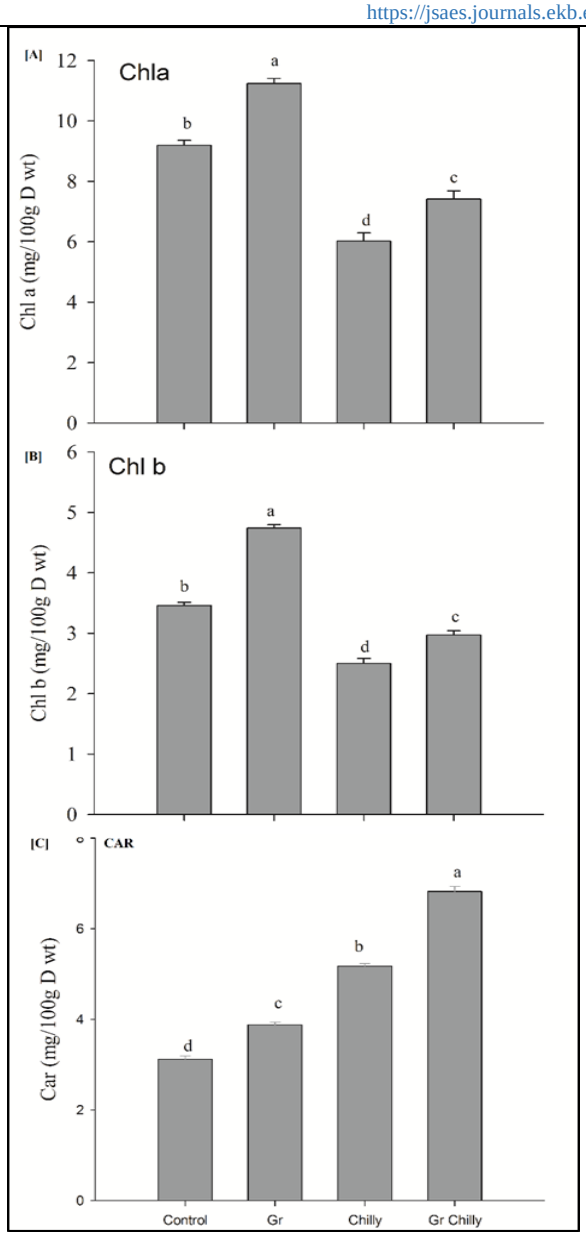

Figure 1. Changes in photosynthesis pigments content in sugar cane variety (Guitang 49) under all experimental treatment. A; Chlorophyll a (Chl a), B; Chlorophyll b (Chl b) and C; Carotenoids (CAR). Diverse letters pointed to the significant differences at $p \leq 0.05$ according to two-way ANOVA and Duncan test analysis

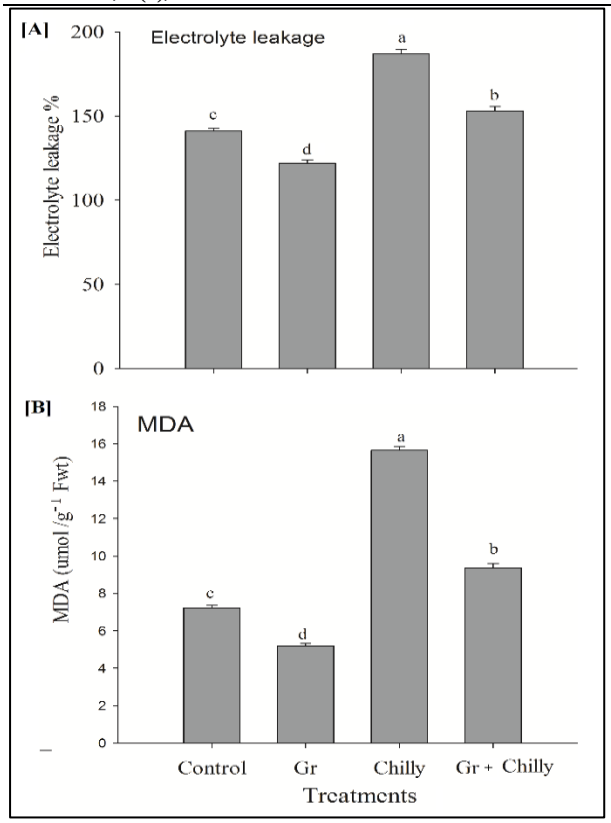

Figure 2. Changes in MDA content and rate of electrolyte leakage in sugar cane, cultivar (Guitang 49) under all experimental treatment. Diverse letters point to the significant differences at $p \leq 0.05$ according to two-way ANOVA and Duncan test analysis.

Table 1. Summary of transcriptome data, GC ratio, Q30% and error %.

sample	R	Clean Reads	GC (%)	Q30 (%)	Error (%)
Control	1	54695496	54.14	94.79	0.02
	2	57090582	53.6	94.62	0.02
	3	45289564	53.57	95.09	0.02
mean		52358547	53.77	94.83	0.02
Ch	1	46034864	54.12	94.83	0.02
	2	54633564	54.28	95.04	0.02
	3	47440170	53.87	94.37	0.02
mean		49369533	54.09	94.74	0.02
G	1	56966640	54.02	94.4	0.02
	2	60517394	53.65	94.69	0.02
	3	52216370	53.36	94.22	0.02
mean		56566801	53.67	94.43	0.02
G+Ch	1	44523910	53.03	94.31	0.02
	2	47433660	53.79	94.47	0.02
	3	44770396	53.37	94.86	0.02
mean		45575929	53.39	94.54	0.02

3.4. Function annotation of unigenes

All transcripts and unigenes were annotated based on 7 different databases as summarized in figure 3. Where 49 % were annotated in Nr (NCBI non-redundant protein sequences), 74.2 % in Nt (NCBI non-redundant nucleotide sequences), 38.67 % in Pfam (Protein family), 12.09 % in KOG/COG (Clusters of Orthologous Groups of proteins), 35.53 % in Swiss-Prot (A manually annotated and reviewed protein sequence database),16.56 % in KO (KEGG Ortholog database) and 38.67 % in GO (Gene Ontology). Unigenes and transcripts length ranged from 300 to >200 bp (Figure 4A). Distribution of unigenes under biological process, molecular function and cellular component through GO enrichment analysis produced 56 functional groups 26 groups categorized as BP, 20 groups as CC and 10 groups as MF (Figure 4 B). Meanwhile, the KEGG enrichment analysis classified the unigenes into 19 functional pathways (Figure 4C) involved; environmental adaptation, nucleotide metabolism, overview, glycan biosynthesis and metabolism, metabolism of terpenoids and polyketides, lipid metabolism, metabolism of cofactors and vitamins, energy metabolism, carbohydrate metabolism, amino acid metabolism, biosynthesis of other secondary metabolites, translation, transcription, replication and repair, folding ,sorting and degradation, signal transduction, membrane transport and catabolism pathways. Also, KOG enrichment analysis pointed to appearance of 26 functional pathways classified from A to Z (Figure 4 D).

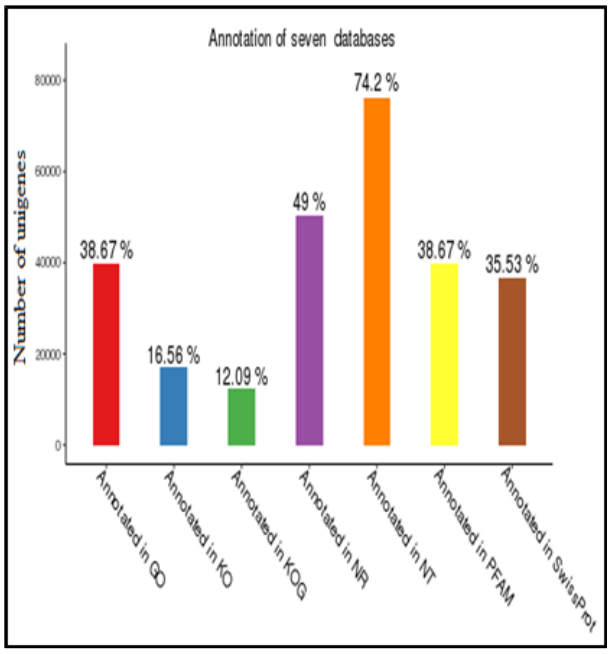

Figure 3. Percentage of unigenes annotated against used software

3.5. Identification and functional annotation of differentially expressed genes (DEGs) under different treatment combinations

To investigate the effects of chilly stress and the recovery effect of GrNPs on sugarcane from a genetic viewpoint, differentially expressed genes (DEGs) were detected at pairwise comparison such as chilly vs control

(Ch vs control), chilly vs grapheme (Ch vs Gr), grapheme + chilly vs grapheme (Gr+Ch vs Gr) and chilly vs graphene+ chilly (Ch vs Gr+Ch) (Figure 5). In total, 169 DEGs, including 63 up- and 106 down regulated genes were screened out in Ch vs. control comparison (Figure 5A). In Ch vs Gr comparison 7075 DEGs containing 2718 up and 4357 down regulated genes were detected (Figure 5B). In Gr+Ch vs Gr comparison, 542 DEGs including 43 up and 499 down regulated genes were detected (Figure 5C). In Ch vs Gr+Ch comparison, 2578 DEGs including 744 up and 1834 down regulated genes were screened out (Figure 5D). Considering recovery signs observed after graphene treatment on photosynthesis and membrane stability (Figure 1 and 2), identified DEGs in both Gr and G +Ch treatment could be expected to play significant roles in the regulation of chilling tolerance.

To understand the biological functions of identified DEGs in each treatment group, we performed gene ontology (GO) enrichment analysis as a systematic way to interpret and understand the functional implications of changes in gene expression. For the comparison of Ch vs control (Figure 6A), the 4422 DEGs were distributed into 56 functional groups. These functional groups distributed on three categories: the first is biological process (BP) which involved 20 groups, groups of the metabolic process were the most abundant. The second category is cell component (CC) that compressed of 16 functional groups, groups of transcription factor complex were the most denoted groups. The third category is molecular function (MF) that involved 20 functional groups, groups of catalytic activity function represented the most abundant among the 20 functional groups. For the comparison of Ch vs Gr (Figure 6B), the 154677 DEGs were divided into 51 functional groups distributed among the main three categories. BP category comprised of 20 functional groups with similar abundance. CC category involved 11 functional groups, membrane groups were the most abundant. Molecular function category contained 20 functional groups, catalytic activity groups were the most represented groups. The lowermost number of functional groups was identified in the comparison of Ch vs Gr+Ch (Figure 6C). 58565 DEGs were divided into 20 functional groups distributed on three categories, BP category involved 8 functional groups the most abundant was single-organism process. CC category showed two functional groups anchored component of plasma membrane and transcriptional factor complex. MF category consists of 10 functional groups; catalytic activity was the most abundant. The 12174 DEGs in the comparison of Gr+Ch vs Gr showed 36 functional groups divided to 20 in BP category, 7 in CC category and 9 in MF category with abundance of protein complex function groups in CC category and nucleic acid binding transcription factor and transcription factor activity sequence groups in MF category (Figure 6D). Overall, present analysis points to the important of GO terms of transcription factor complex, catalytic activity, membrane properties and single-organism process to be considered in improving sugarcane tolerance to cold temperature. Transcription factors play critical functions in the regulation of plant responses via targeting the related genes at the expression level to stimulate adaptation for different stresses (Huang et al., 2022). Role of cell membrane system-related genes were reported to be elaborate in the acquisition of cold tolerance in sugarcane plants (Huang et al., 2022).

KEGG pathway enrichment analysis was conducted to all annotated transcript based on KOBAS software to obtain a deeper understanding of the functions of DEGs involved in tested treatment. The top 20 enriched pathways from all detected pathways in the four comparison groups were detected (Figure 7). Phenylpropanoid biosynthesis (ko00940) was a common pathway represented in all comparison groups. For the Ch vs. control comparison, 37 DEGs were mapped into 21 KEGG pathways, 36 of them were involved in the top 20 pathways where phenylpropanoid biosynthesis (ko00940) and phenylalanine metabolism (ko00360) were highly enriched KEGG pathways (Figure 7A). For the comparison of Ch vs Gr the 1553 DEGs were mapped into 118 KEGG pathways, 583 of them were involved in the top 20 enriched pathways (Figure7B). In Ch vs. Gr+Ch comparison 523 DEGs were mapped into 102 KEGG pathways while 234 DEGs were involved in the top 20 KEGG pathways (Figure 7C). For Gr+Ch vs Gr comparison, 105 DEGs were mapped into 48 KEGG pathways, the top 20 KEGG pathways involved 65 of them (Figure 7D).

The KEGG analysis stated that altered number of annotated DEGs were differentially up- and down-regulated among all treated combinations. The most enriched up and down regulated KEGG were summarized for all treatment comparisons (Table 2). The commonly enriched KEGG pathways that were detected in almost all treated groups are: plant hormones signal transduction, phenylpropanoid biosynthesis, starch and sucrose metabolism, carotenoid biosynthesis and photosynthesis antenna proteins (Figure 7, Table 2).

Plant hormones are important and vital agents employed in normal and fruitful growth and development of plants particularly against negative environmental surroundings (Kong et al., 2020). The obtained results showed that about 140 annotated DEGs were intricate in the regulation of hormones signaling mechanisms in sugarcane under chilly stress and application of Gr NPs (Fig. 7 and Table 2). The Ch vs Gr treated group showed 67 highly downregulated DEGs while 20 upregulated DEGs and Ch vs Ch + Gr treated group revealed 35 downregulated and 8 highly upregulated DEGs intricate in plant hormone signal transduction pathway. While, Gr+Ch vs Gr treated groups have shown only 10 downregulated DEGs.

Phenylpropanoids are bioactive secondary metabolites synthesized from the essential amino acid phenylalanine. They are very critical for the biosynthesis of numerous compounds such as: flavonoids, lignins, coumarins, and lignans (Fraser and Chapple, 2011). Thus, Phenylpropanoids represent important roles in plant development and responses to different environmental stimuli. It was reported that phenylpropanoid compounds func-

tion as powerful antioxidants (Rahim et al., 2023). In present results, 99 annotated DEGs were intricate in the regulation of phenylpropanoid biosynthesis pathway in sugarcane under chilly stress and application of Gr NPs (Figure 7 and Table 2).

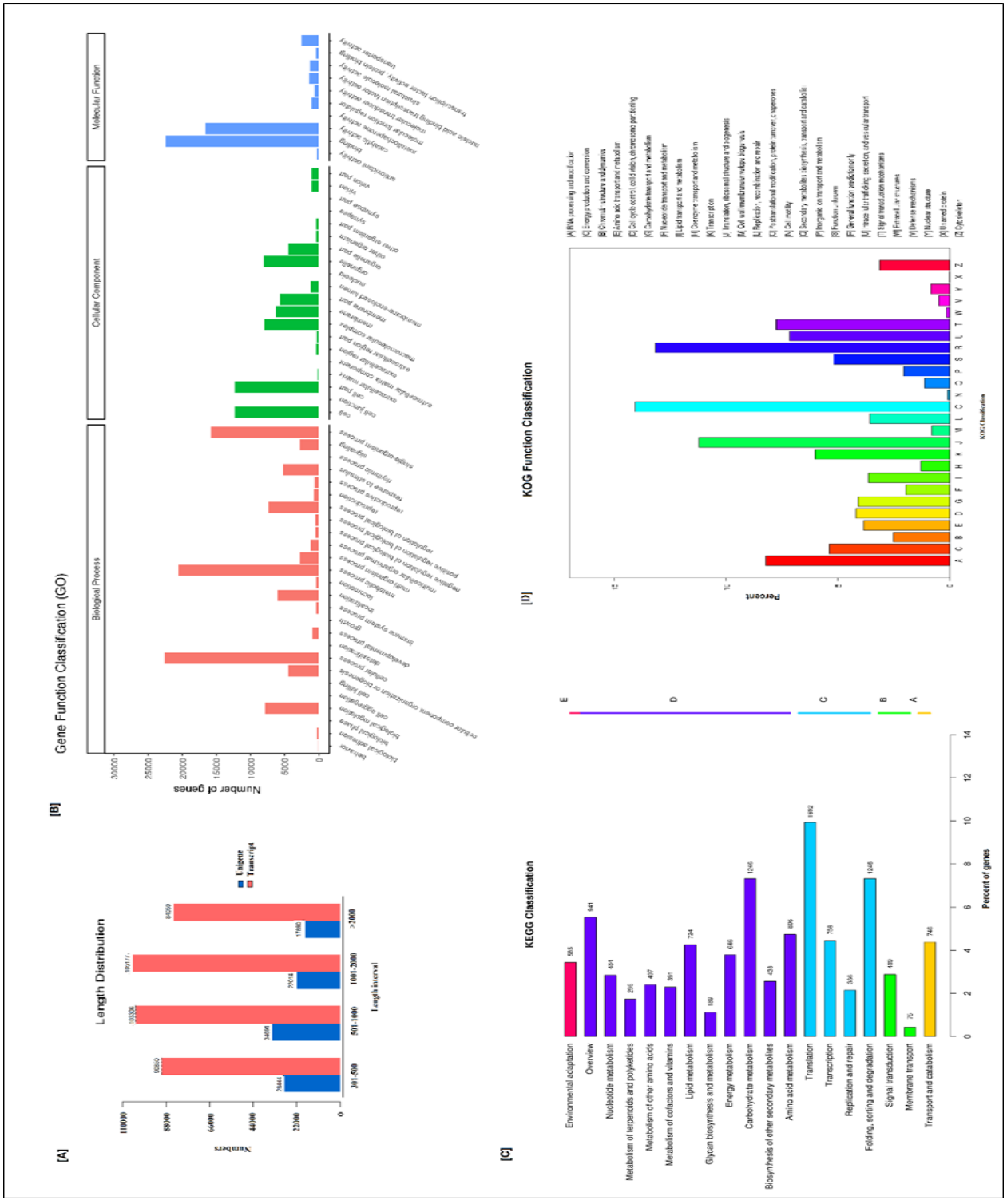

Figure 4. A: length distribution of annotated DEGs, B: GO enrichment analysis of all annotated DEGs for the main three categories, Biological process(Bb), cell components (CC)and molecular function(MF); C: KEGG classification for main functional pathways; D: KOG classification for main functional pathways annotated to eukaryote-specific version of the Clusters of Orthologous Groups (COG**).**

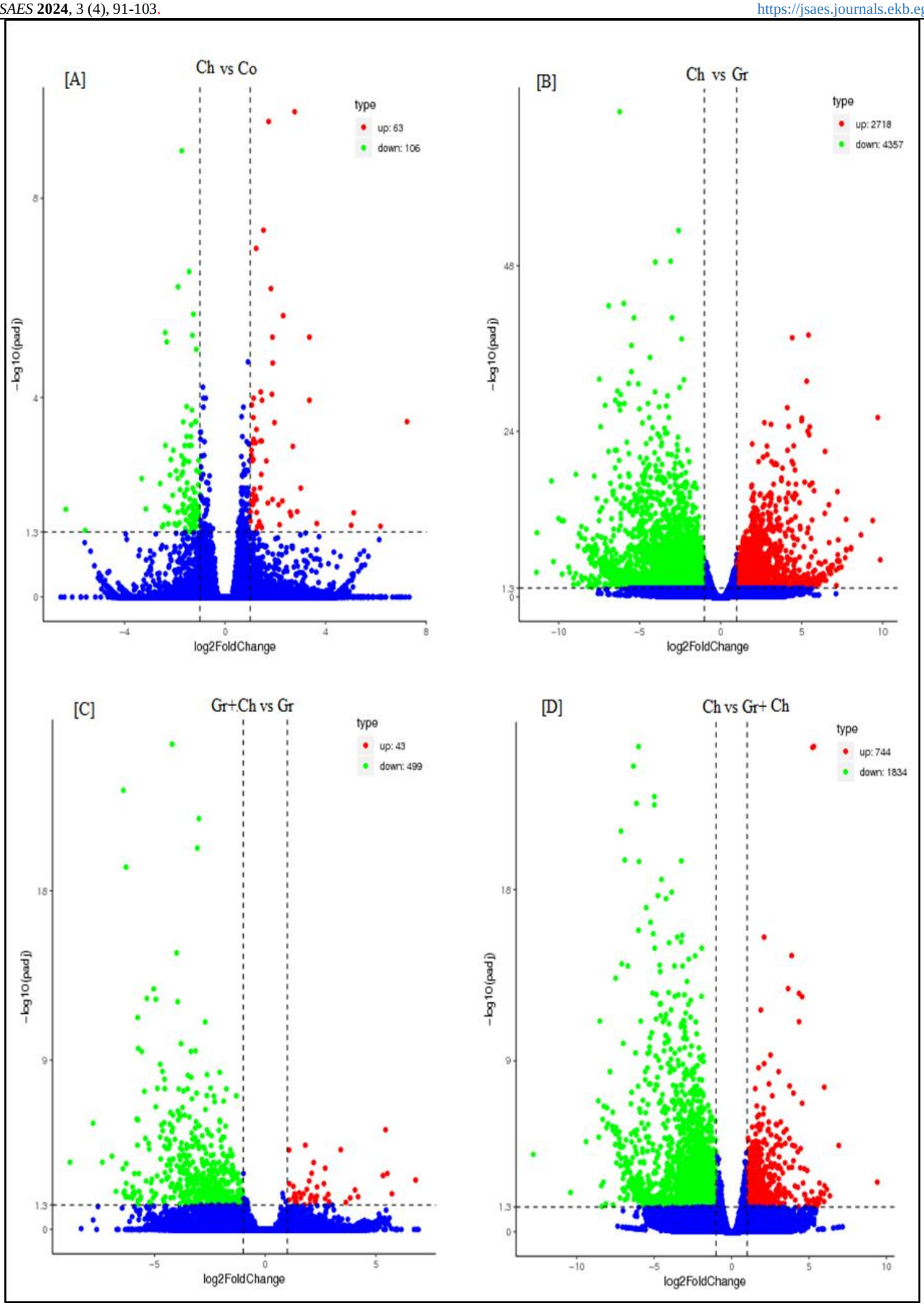

Figure 5. Numbers of DEGs in tested variety of sugar can (Guitang 49) at each comparable group. Ch vs Control [A], Ch vs Gr [B], Ch vs Gr+Ch [C] and Gr+Ch vs Gr [D] The up- and downregulated DEGs were displayed.

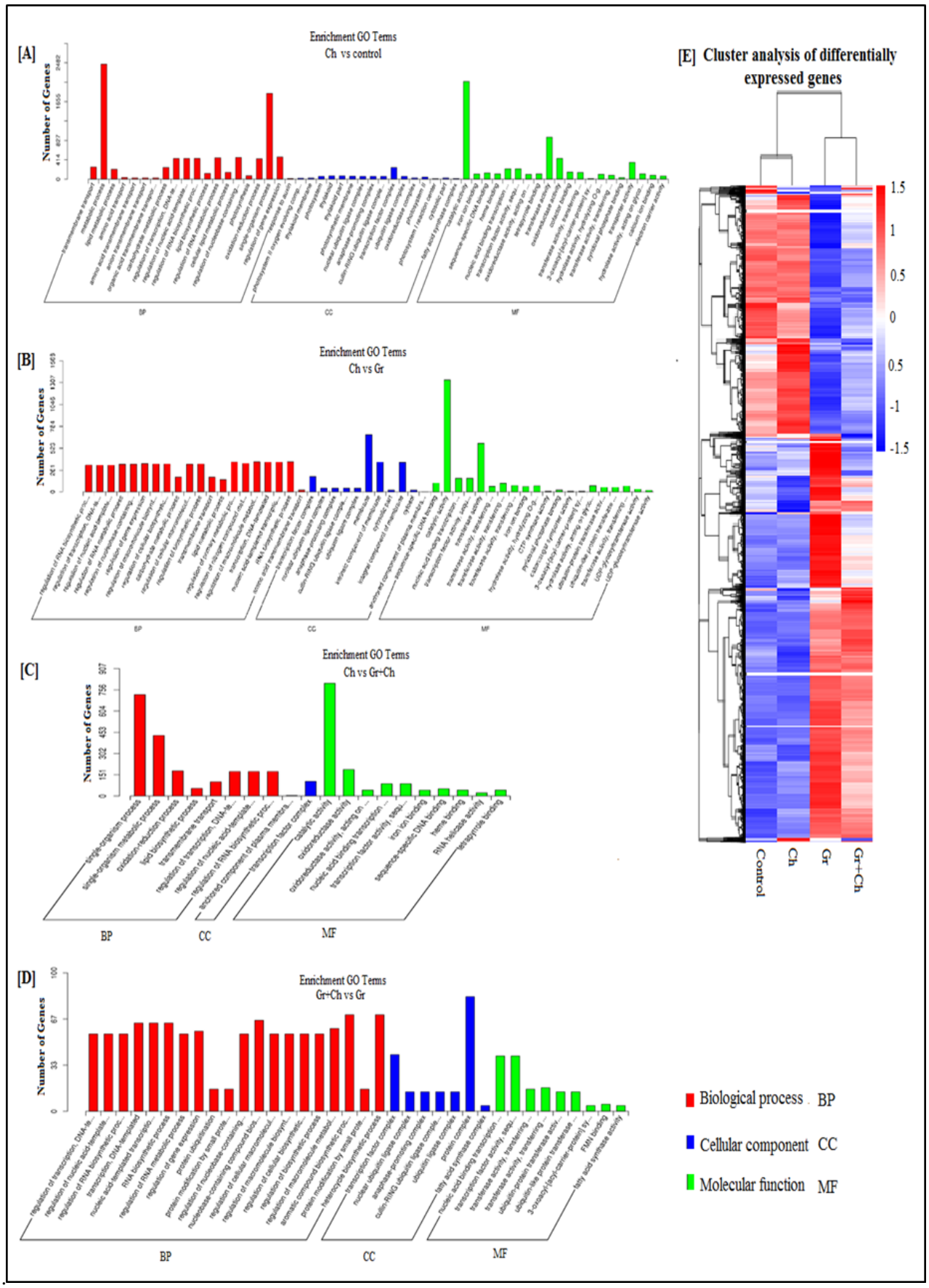

Figure 6. A-D: Gene ontology (GO) sorting of all DEGs in all treatment comparison into the three categories: Biological Process (BP), Cellular Component (CC), and Molecular Function (MF). E; Hierarchical cluster heat map analysis of the transcription patterns of all treatment related DEGs at different experimental conditions.

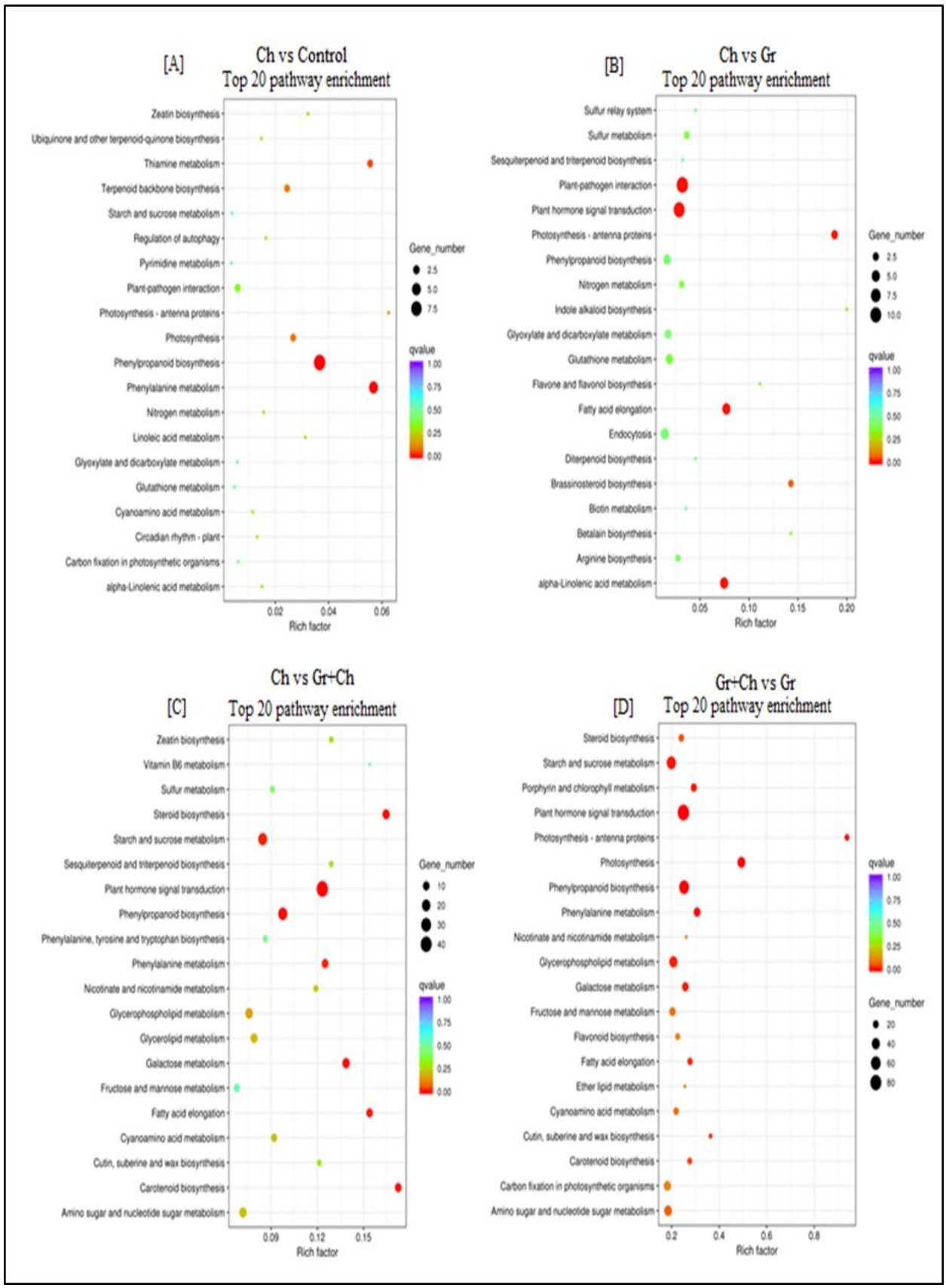

Figure 7. KEGG pathway enrichment analyses of DEGs in Ch vs. Control [A], Ch vs. Gr [B], Ch vs Gr+Ch [C] and Gr+Ch vs Gr [D]. Left Y-axis represent the KEGG pathways. The X-axis displays the enrichment factor. The size and color of each point denote the number of genes enriched in a precise pathway.

Table 2. The most enriched up and down regulated KEGG pathways in all treatment comparisons

comparison pair	Up regulated KEGG		Down regulated KEGG	
	pathway	Gene number	pathway	Gene number
Ch vs control	Thiamine metabolism	2	Phenylpropanoid biosynthesis	8
	Photosynthesis	2	Phenylalanine metabolism	5
	Terpenoid backbone biosynthesis	2	Photosynthesis - antenna proteins	1
	Regulation of autophagy	1	Zeatin biosynthesis	1
	Circadian rhythm - plant	1	Nitrogen metabolism	1
Ch vs Gr	Photosynthesis	34	Plant hormone signal transduction	67
	Photosynthesis - antenna proteins	15	Phenylpropanoid biosynthesis	45
	Porphyrin and chlorophyll metabolism	18	Galactose metabolism	22
	Ribosome	56	Cutin, suberine and wax biosynthesis	12
	Carbon fixation in photosynthetic organisms	18	Starch and sucrose metabolism	40
	Tryptophan metabolism	12	Glycerophospholipid metabolism	31
	Glyoxylate and dicarboxylate metabolism	15	Plant-pathogen interaction	46
	Tyrosine metabolism	8	Phenylalanine metabolism	18
	Pentose phosphate pathway	12	Carotenoid biosynthesis	14
	Zeatin biosynthesis	5	Nicotinate and nicotinamide metabolism	11
	Phenylalanine metabolism	9	Amino sugar and nucleotide sugar metabolism	28
Ch vs Gr+Ch	Sulfur metabolism	4	Plant hormone signal transduction	35
	Zeatin biosynthesis	3	Steroid biosynthesis	12
	Thiamine metabolism	3	Carotenoid biosynthesis	10
	Pantothenate and CoA biosynthesis	3	Phenylpropanoid biosynthesis	20
	Pentose and glucuronate interconversions	4	Galactose metabolism	12
	Monobactam biosynthesis	2	Fatty acid elongation	9
	Biosynthesis of unsaturated fatty acids	3	Phenylalanine metabolism	10
	Biotin metabolism	2	Glycerophospholipid metabolism	15
	Tyrosine metabolism	3	Starch and sucrose metabolism	18
	Plant hormone signal transduction	8	Amino sugar and nucleotide sugar metabolism	13
	Fructose and mannose metabolism	4	Glycerolipid metabolism	11
Gr+Ch vs Gr	Photosynthesis - antenna proteins	3	Plant-pathogen interaction	11
	Fatty acid elongation	2	alpha-Linolenic acid metabolism	5
	Diterpenoid biosynthesi	1	Plant hormone signal transduction	10
			Brassinosteroid biosynthesis	2
			Fatty acid elongation	3
			Indole alkaloid biosynthesis	1
			Betalain biosynthesis	1

The Ch vs control treated group revealed 8 highly downregulated DEGs and 1 upregulated DEGs while in Ch vs Gr treated group 45 highly downregulated DEGs and 17 upregulated DEGs were annotated. Ch vs Ch + Gr

treated group showed 20 downregulated and 4 highly upregulated DEGs elaborated in phenylpropanoid biosynthesis pathway. While Gr+Ch vs Gr treated groups have shown only 4 downregulated DETs.

Starch represents the key energy source for plants and are elaborated in the mechanisms of abiotic stress responses (Deng et al., 2020). Starch can be decomposed into small carbohydrate molecules to release energy and maintain stability in the osmotic system (Deng et al., 2020). The obtained results revealed that 73 DEGs were annotated into starch and sucrose metabolism pathway (Figure 7 and table 2). 14 DEGs were up regulated in Ch vs Gr treated group while 4 DEGs were downregulated in the same group. In both of Ch vs Gr+ Ch and Gr vs Gr+Ch treated groups, 19 DEGs of starch and sucrose metabolism were down regulated. Starch hydrolysis in starch and sucrose pathway was reported to be one of survival tactics in *A. vulgaris* under abiotic stress (Chen et al., 2023).

Photosynthesis process is one of the most essential tasks on Earth. Photosynthetic light-harvesting antennae are pigment-binding proteins that catch light and convey energy that enables life in our planet (Arshad et al., 2022). In the present results, 29 DEGs were annotated into photosynthesis antenna proteins pathway. 15 DEGs were upregulated in Ch vs Gr treated group. While 3 were up regulated and 11 were downregulated in Gr+Ch vs Gr treated group. Also carotenoids are considered as main component in photosynthesis process. Carotenoid biosynthesis pathways play important roles in plant growth and significantly elaborated in the photosynthesis process as well as their role in protection chlorophyll from photo-oxidative damage (Howitt and Pogson, 2006), Twenty-six DEGs were annotated into carotenoids biosynthesis pathways where 24 DEGs were downregulated in both Ch vs Gr and Ch vs Gr+Ch treated groups (Figure 7 and table 6).

Obtained results showed the positive role of GrNPs on chilly-stressed plants. Treatment with GrNPs resulted in improving plant growth and acquisition of chilly tolerance in sugarcane. Determined physiological parameters point to the positive effect of GrNPs on membrane stability as showed in the reduction of rate of membrane leakage and MDA content. That indicates the minimizing of chilly oxidative stress on stressed plants treated with GrNPs. Gene ontology and enrichment analysis for differential expressed genes among all treated groups showed changes in the expression pattern as up and down regulation of some main function pathways in correlation with chilling and GrNPs. Considering the commonly enriched KEGG pathways that were found in almost all treated groups, plant hormones signal transduction, phenylpropanoid biosynthesis, starch and sucrose metabolism, carotenoid biosynthesis and photosynthesis antenna proteins could be useful to understand the mechanisms underlying the response to graphene and could be used for further confirmation of effect of Gr-NPs on ameliorating negative effects of chilly stress on different plants.

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