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Whole-Transcriptome Responses to Environmental Stresses in Agricultural Crops Treated with Carbon-Based Nanomaterials

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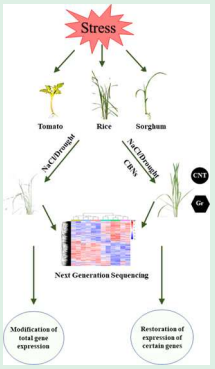
ABSTRACT: Carbon-based nanomaterials (CBNs) such as carbon nanotubes (CNTs) and graphene can be beneficial to crops exposed to abiotic stresses such as drought and high salinity. Our findings suggest that the improvement observed in stressed crops treated with CBNs can be associated with CBN-induced restoration of gene expression. When subjected to salt stress, sorghum seedlings showed modified expression in 51 stress-related genes. The introduction of CNTs or graphene into the salty growth medium resulted in the restoration of the expression of 29 affected genes, resembling that of untreated sorghum seedlings. RNA-Seq approach allowed us to analyze the total gene expression of CBN-treated rice exposed to water-deficit stress and gene expression of CBN-treated tomato plants exposed to salt stress. The application of CNTs or graphene resulted in full or partial restoration of expression of 458 and 1620 genes, respectively, affected by water-deficit stress in rice. Similarly, CBN treatment of NaCl-exposed tomato seedlings led to full or partial restoration of 1639 and 1391 salt-affected transcripts, respectively. Of the genes with restored expression, many of them were identified as major stress-response genes and major transcriptional factors (aquaporins, dehydrins, and heat shock proteins/co-chaperons, *NAC*, *WRKY*) and were associated with key stress-signaling pathways (ABA-signaling, $InsP_3$ signaling, and MAPK signaling) in all three tested plant species. These findings provide evidence that CBNs can provide halotolerance and drought tolerance by normalizing the expression of affected stress genes.



KEYWORDS: *carbon-based nanomaterials, environmental stress, gene expression, salt tolerance, drought tolerance, RNA-Seq*

INTRODUCTION

Carbon-based nanomaterials (CBNs) can affect plants in a dose-dependent manner; CBNs can be toxic in high concentrations,[1] but can positively regulate seed germination and plant growth/development in low and average doses.[2−9] In order to understand the underlying mechanisms governing these dose-dependent effects, the impact of CBNs on the plant transcriptome, metabolome, and proteome must first be characterized. Previously, multiwalled carbon nanotube (MWCNT) enhancement of seed germination and growth was linked to the regulation of plant stress genes, particularly the upregulation of water channel genes (aquaporins) in CNT-treated seeds and plant organs.[2,3,5,7] More recently, Pandey et al. (2018) demonstrated that the addition of CNT and graphene to a salty growth medium can significantly reduce symptoms of salt stress in sorghum and switchgrass.[10] The authors suggested that the effect of CBNs on the elimination of environmental stress symptoms *in planta* can be explained by two possible mechanisms: interactions of CBNs with harmful ions and influence of CBNs on the expression of plant genes involved in stress response.[10] Expression of sorghum aquaporins was stimulated by the application of CNT and graphene in salt-stressed sorghum plants.[10] The ability of CBNs to regulate the response of plants to environmental stress is intriguing as CBNs might offer promise in mitigating agricultural losses to abiotic stress. Abiotic stress factors provide the largest impact on crop productivity, dramatically reducing the yield of major agricultural species worldwide.[11] A simple application of low-dose CBNs to seed/plant tissues could be a reasonable alternative to genetic manipulations toward the improvement of plant stress tolerance. Before CBNs can be used in agricultural applications, the mechanisms of CBN-associated restored expression need to be well understood. Previously, a microarray analysis of tomato plants exposed to CNT revealed that CNT can affect multiple branches of plant stress signaling at the level of the transcriptome.[2] This work aims to identify the changes CBNs induce in plants at the transcriptome level when grown under drought or salty conditions. To achieve this, transcriptome analysis of tomato, rice, and sorghum grown under drought or salt stress conditions in the absence or presence of CNTs was completed using real-time PCR and RNA-Seq.

The findings of this study contribute to the understanding of the biomechanisms of CBNs *in planta* and emphasize the

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importance of characterization of the influence of nanomaterials on "omics" levels.

MATERIALS AND METHODS

CBNs and Plant Seeds Used for Experiments. Commercially available CBNs were used for the study. Multiwalled CNTs (MWCNT−COOH, OD 13−18 nm; length 1−12 μm) and graphene nanoplatelets (<3 layers; lateral dimension 1−2 μm) were purchased from Cheap Tubes (Brattleboro, VT). CNTs and graphene were characterized and checked for purity with transmission electron microscopy and Raman spectroscopy.[7] Endotoxins latent in the CBNs were eliminated by autoclaving the solutions three times at 121 °C, 15 lb/in.^{2} pressure for 20 min, as described by Lahiani et al.[8] Tomato (*cv. Micro-Tom*) was obtained from Reimer Seeds Co. Inc., MD, USA. Rice grains (*cv. Nipponbare*) were provided by the University of Arkansas System, Agricultural Division.

Cultivation of Plants and Stress Experiments. Tomato seeds were sterilized by washing with 70% ethanol for 2 min, followed by a rinse with double distilled water, then bathed in a 50% bleach solution, and vortexed for 30 min. After sterilization, seeds were rinsed nine times with sterile water. For salt stress experiments involving tomato plants, 120 tomato seeds per treatment were mounted on either Murashige and Skoog (MS) medium, MS medium supplemented with 100 mM of NaCl, MS medium supplemented with 100 μg/mL CNT, MS medium supplemented with 100 μg/mL graphene, MS medium supplemented with 100 mM NaCl + 100 μg/mL of CNT, and MS medium supplemented with 100 mM NaCl + 100 μg/mL of graphene. Seeds were incubated at 24 °C, with 12 h day photoperiods and light intensity of 105 μmol/s·m^2 in a growth chamber for 21 days. Shoot and root length of germinated tomato seedlings were recorded at the end of the experimental period for each treatment. For all experiments involving rice plants, rice grains were surface sterilized by chlorine gas in a desiccator and germinated on MS *in vitro*. 3 week old rice seedlings were transferred to the soil and grown under a 14 h light/10 h dark cycle at 28 °C in a greenhouse. Treatment of rice plants with CBNs was carried out with and without imposed water-deficit stress. For regular watering conditions, a 100 mL solution of graphene or CNT solution (200 μg/mL) was added to 8 week old rice plants once a week for four consecutive weeks in each experimental pot containing 400 g of soil. The final amount of CBNs in each pot before the water-deficit stress experiment was 80 mg of CNTs or graphene per 400 g of soil. For control (untreated) plants, 100 mL of deionized water was applied at the same time. An equal amount of pure water was used for regular irrigation of control and CBN-treated plants daily. For water-deficit conditions, 12 week old rice plants treated with CBNs, as described below (six plants per treatment), were not exposed to any watering practice for 6 weeks. During the drought stress experiment, the volumetric water content of the soil in pots was measured at 0, 3, 5, and 7 days using the ProCheck Decagon Device (Decagon Devices, Inc., USA). The relative water content (RWC) of the leaves was measured by the whole leaf method during drought stress.[12] After the whole drought period (41 days), the seed yield was measured in control plants and plants treated with CBNs.

Real-Time PCR of Sorghum Seedlings Exposed to Salt Stress. Total RNA was isolated from 10 days old control (untreated) seedlings, seedlings exposed to 100 mM NaCl, 100 mM NaCl + 100 μg/mL of CNT, and 100 mM NaCl + 100 μg/mL of graphene plants by using the RNeasy Plant Mini Kit (Qiagen Inc. Valencia, CA). Exposure of seedlings to stress and CBNs was performed by introduction of NaCl and CBNs into agar growth medium used for germination and development of sorghum seedlings for 10 days. Details of the salt stress experiment involving sorghum seedlings were described previously.[10] The synthesis of cDNA for all generated RNA samples was carried out using a SuperScript III First-Strand Synthesis System Kit (Invitrogen, Carlsbad, CA) with dT16-oligonucleotide primers, according to the manufacturer protocol. The cDNA was utilized for the real-time PCR analysis of different classes of osmotically induced genes. Gene-specific primers used for real-time PCR are presented in Table S1. Actin and 18S rRNA gene were used as the internal control for expression analysis.

RNA-Seq of Tomato and Rice Seedlings Exposed to Abiotic Stresses. RNA-Seq total RNA was isolated from 21 days old tomato seedlings grown on MS medium, MS medium supplemented with 100 mM of NaCl, MS medium supplemented with 100 μg/mL CNT, MS medium supplemented with 100 μg/mL graphene, MS medium supplemented with 100 mM NaCl + 100 μg/mL of CNT, and MS medium supplemented with 100 mM NaCl + 100 μg/mL of graphene. For rice RNA-Seq, total RNA was isolated from the leaves of rice plants exposed to regular watering (control), from plants treated with CNT and graphene under regular watering, and from plants exposed to 25 days of water-deficit stress in the presence of CNT added to soil mix (80 mg of CBN per 400 g of soil). The total RNA from tomato and rice samples was extracted using the RNeasy Plant Mini Kit (Qiagen Inc. Valencia, CA). The quality of RNA was measured using ND-2000 (NanoDrop Technologies). RNA samples of both species were sent to Novogene Co., Ltd. (Sacramento, CA) for RNA-Seq. RNA-Seq data analysis was provided by Novogene Co., Ltd., which included the sample quality control, library construction, library quality control, sequencing, data quality control, and bioinformatics analysis. RNA sequencing was performed via Illumina platforms (Illumina, San Diego, CA).

Validation of RNA-Seq Analysis in Tomato and Rice Plants. RNA was isolated from tomato plants exposed to salt stress and rice plants exposed to water-deficit stress, as described above. The cDNA was generated from 1 μg of total RNA using a SuperScript III First-Strand Synthesis System (Invitrogen, USA) with a dT_{20} oligonucleotide as a primer, according to the manufacturer protocol. cDNA samples were diluted and used for real-time quantitative PCR analysis with SYBR Green PCR master mix (Thermo Fisher Scientific, UK) in an iCycler iQ Multicolor Real-Time PCR detection system (Bio-Rad, USA). For rice genes, aquaporin, *OsPIP1-1* (XP_015636702.1), and LRR receptor-like serine/threonine protein kinase, *OsSIK-1* (XP_015641250.1), were amplified. To amplify *OsPIP1-1*, 5′-TGATCTTCGCGCTCGTCTAC-3′(forward) and 5′-GACCAG-GAACACCGCAAAAC-3′(reverse) primers were used. To amplify *OsSIK1*, 5′-CTGGCCCGTGGTACTTTGAT-3′(forward) and 5′-AGAGCTTGCATCAGGCCAAT-3′(reverse) primers were used. To amplify the tomato gene for pathogenesis-related protein 1 (PR1) (NM_001247429.1) 5′-TGGACGTTGTCCTCTCCAGT-3′ (forward) and 5′-CGTCTTGGTTGTGCTAGGGT-3′ (reverse) primers were used. House-keeping gene (18S) was amplified for all samples using primers: 5′-AGGCCGCGGAAGTTTGAGGC-3′ and 5′-ATCAGTGTAGCGCGCGTGGG-3′.

Three independent biological replicates were used in the analysis. For each biological replica, three technical replicas were run. The real-time PCR data were analyzed by the "comparative count" method to obtain relative mRNA expression of each tissue, as described in the iCycle manual (Bio-Rad).

RESULTS AND DISCUSSION

CBNs Influence the Expression of Selected Osmotically Induced Genes in Young Seedlings of Sorghum Exposed to NaCl. Previously, we have reported that CBNs can reduce symptoms of toxicity caused by salt stress in common bioenergy crops, specifically sorghum and switchgrass.[10] The introduction of MWCNTs or graphene into agar growth medium supplemented with NaCl reduced inhibition of seed germination of switchgrass and suppression of shoot and root growth of switchgrass seedlings caused by salt stress.[10] It is logical to assume that CBNs can affect the expression of multiple genes and branches of plant stress signaling. To better understand the molecular basis for the improvement of abiotic stress tolerance in plants exposed to CBNs, we investigated the effects of CNTs and graphene on the stress-responsive gene network in sorghum seedlings exposed to salt stress. Different types of genes that are

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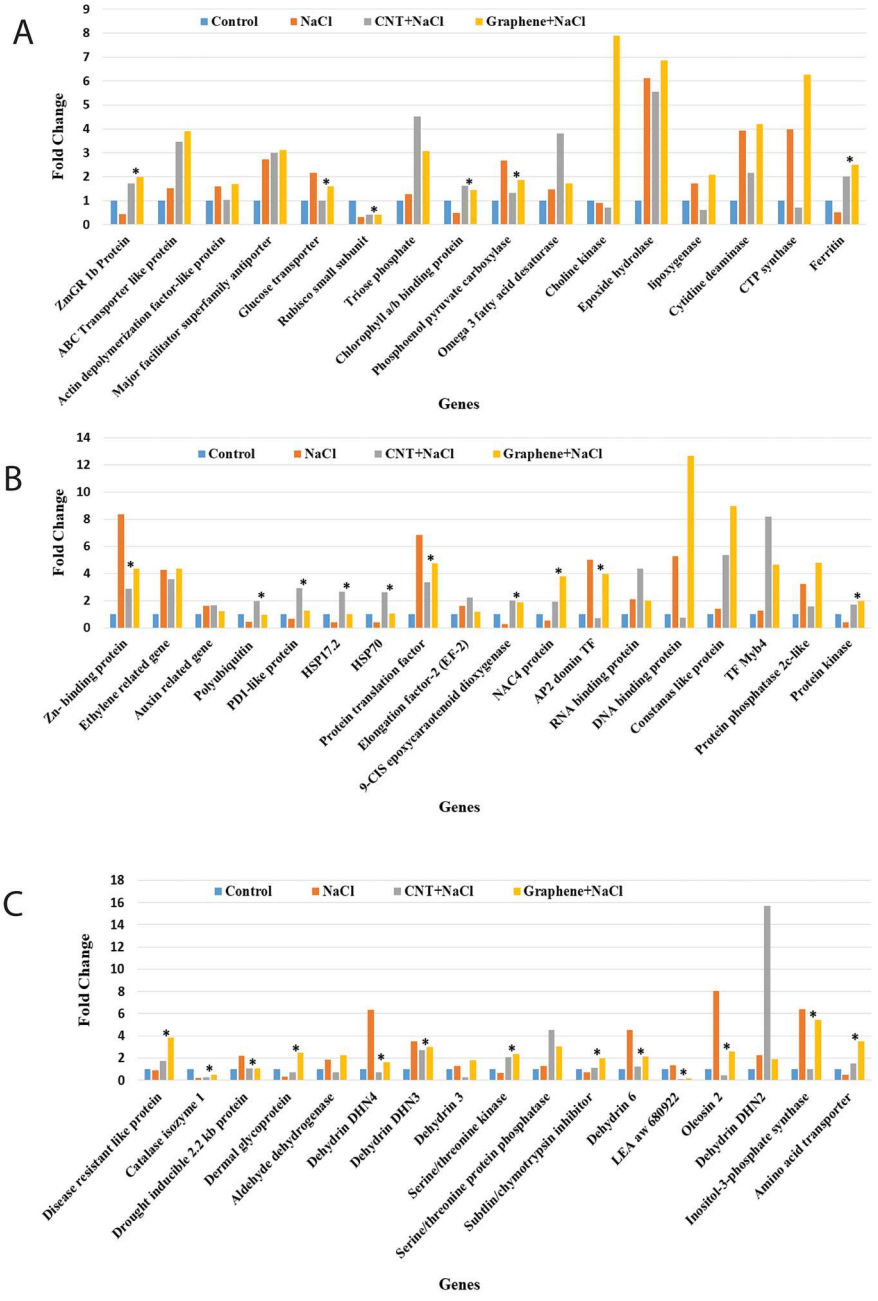

Figure 1. (A B and (C). Effects of CBNs on the expression of selected osmotic, stress-induced genes in sorghum exposed to NaCl (100 mM). The normalization of gene expression was defined as change in expression compared to that of control (untreated) sorghum plants. The level of expression in the control plant was recognized as 1. Changes of expression in NaCl and CBN-treated plants were calculated against the control level for each gene. Asterisks denote the significant change in fold for both treatments [(CNT + NaCl and graphene + NaCl), compared to NaCl treatment ($p < 0.05$)].

responsible for adaptation under harsh environmental conditions have been identified in sorghum.[10,13] Moreover, transcriptomic profiling of osmotically induced genes in sorghum was performed by Buchanan et al.[14] In detail, Buchanan et al. performed the functional classification using BLASTX and identified four different categories of osmotically induced genes: genes encoding proteins that are responsible for the regulation of gene expression; genes involved in stress signaling (abiotic and biotic stress signaling); genes that are responsible for growth and metabolism; and genes with unknown functions.[14] Using the microarray technology, Buchanan et al. analyzed 12,982 unique gene clusters in sorghum exposed to 3 and 27 h at osmotic stress.[14] The authors reported that the expression of ∼2200 genes was modified in response to ABA, NaCl, and PEG-mediated dehydration.[14] Exact changes in sorghum stress-responsive genes (333 genes) were studied by real-time PCR analysis.[14] Here, we selected 51 different genes from the pools of osmotically induced genes, as documented by Buchanan et al.,[14] and measured the expression of selected genes in untreated sorghum seedlings, seedlings exposed to NaCl, and seedlings exposed to NaCl and CBNs by real-time PCR. The application of NaCl to sorghum seedlings led to upregulation or downregulation of all 51 selected sorghum stress-responsive

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Figure 2. Phenotypes of 21 day old tomato seedlings germinated on MS medium (control), MS medium supplemented with 100 mM NaCl, MS medium supplemented with CNTs or graphene, and MS medium supplemented with CNT and graphene in the presence of 100 mM of NaCl.

genes (Figure 1). However, the addition of CBNs significantly reduces the changes in gene expression caused by the salt stress and led to the partial or full restoration in expression of several NaCl-affected genes. NaCl affected the expression of all 51 (100%) selected genes, and CBNs were able to modify the expression of 29 stress-related genes (57%), closely resembling those in control plants (Figure 1). These results indicate that the molecular mechanism of positive effects of CBNs on plants is complex and associated with multiple stress signaling pathways and genes. Using the available literature resources, several sorghum genes that were restored to normal expression patterns were analyzed. Real-time PCR analysis revealed that CBNs restored the levels of expression for several dehydrins in sorghum seedlings grown in salty media (Figure 1C).

Dehydrins are an important category of stress-responsive, late embryogenesis abundant (LEA) proteins that are involved in the protection of plants against damage from dehydration, reactive oxygen scavenging, as well as a pathogen or insect diseases.[14] Dehydrins are latent in different plant tissues under normal homeostatic conditions[15] and have been observed to accumulate substantially under stressful conditions. The accumulation of dehydrins can lead to both the cell dehydration that occurs naturally during seed maturation as well as in response to environmental stressors such as high salinity.[16] The roles and mechanisms of some dehydrins *in planta* have not been well characterized fully, but several *in vitro* studies revealed that each dehydrin-encoding gene is related to a specific function.[15] Several investigations into the LEA family of genes suggest that their roles in mitigating abiotic stress relate to their cryoprotective properties,[17] ability to bind lipids,[18] proteins, and metals,[19] and apparent radical scavenging activity.[20] In one genetic engineering study, dehydrin genes were observed in *Physcomitrella patens* to enhance tolerance to osmotic stress, including salt stress.[21] In this study, the exposure of sorghum seedlings to NaCl affected the expression of different types of several dehydrins such as *dehydrin DHN3*, *dehydrin DHN2*, *dehydrin DHN4*, *dehydrin 3*, and *dehydrin 6*. However, the introduction of CBNs into salty medium reversed NaCl-related changes in expression of several genes that encoded dehydrins: *dehydrin DHN4*, *dehydrin DHN3*, and *dehydrin 6* (Figure 1). It may be concluded that CBNs may influence the level of expression of different dehydrin genes, which may in turn affect the downstream dehydrin-mediated signaling pathways *in planta*. It is well documented that plants cope with environmental stress by changing phytohormonal levels, RNA synthesis and RNA stability, translation, protein folding, and post-translational modification.[22−24] Previously, it was reported that osmotic stress can drastically modify the expression of sorghum genes that encode several regulatory proteins.[14] It is well known that NAC transcription factors are plant-specific proteins that are expressed in several tissues.[25] The NAC protein family possesses a wide range of function in the plant such as formation of the shoot apical meristem,[26] flower production,[27] secondary wall formation,[28] leaf senescence,[29] as well as playing a role in biotic and abiotic stress tolerance.[30] Overexpression of NAC transcription factor (ONAC045) in rice has been demonstrated to convey enhanced tolerance to osmotic stress such as salt and drought.[31] In this study, it was observed that the expression of the *NAC4* gene and protein translation factor gene was altered in sorghum plants experiencing salt stress. However, applications of CBNs to the salt-stressed plants led to partial restoration of both the *NAC4* gene and protein translation factor expression levels, as shown in Figure 1B. Heat-shock proteins (HSPs) and aquaporins are two other major groups of stress-responsive plant genes.[32,33] Previously, we have reported that two sorghum aquaporins (*PIP 1;5* and *TIP 1;1*) were down-regulated under salt stress and were restored with CBNs to a similar level of expression exhibited in control plants.[10] In this experiment, it is notable that the expression of sorghum *HSP17.2* and *HSP70* genes also was restored toward the level of untreated control plants by introduction of CNT and graphene to salty medium (Figure 1B).

It may be concluded from these results that the symptoms of salt stress in sorghum may be alleviated by CBNs normalizing the expression levels of several key stress-responsive genes. To investigate whether CBNs may induce expression changes in other crops under similar conditions, RNA-Seq analysis was performed in tomato seedlings grown under salty conditions and seedlings grown with salt and CBNs. To determine whether CBNs can restore the altered gene expression caused by water-deficit stress, RNA-Seq was additionally performed for rice plants exposed to drought and CBNs.

Whole-Transcriptome Responses to Salt Stress in Tomato Seedlings Treated with CBNs. To demonstrate that CBNs can imbue some amount of halotolerance in dicots, as previously seen in monocots,[10] tomato seeds were germinated on either MS medium, MS medium supplemented with 100 μg/mL of CBNs (CNT, graphene), MS medium supplemented with NaCl (100 mM), MS medium containing NaCl (100 mM) in the presence of 100 μg/mL of CNTs or

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Table 1. Phenotypical Analysis of Tomato Seedlings Growing on Medium Supplemented with NaCl (100 mM), CNT (100 μg/mL), Graphene (100 μg/mL), NaCl (100 mM) + CNT (100 μg/mL), and NaCl (100 mM) + Graphene (100 μg/mL)[a]

	length (cm)		total biomass (g)	
	shoot	root	fresh	dry
control	2.27 ± 0.762	3.93 ± 1.385	0.08692 ± 0.03	0.00674 ± 0.002
NaCl (100 mM)	0.23 ± 0.168**	0.45 ± 0.4**	0.00283 ± 0.0025**	0.000255 ± 0.0002**
CNT (100 μg/mL)	3.215 ± 0.374*	4.96 ± 0.556*	0.10863 ± 0.023	0.006475 ± 0.001
graphene (100 μg/mL)	3.73 ± 0.411*	6.645 ± 0.807*	0.118 ± 0.019	0.00833 ± 0.001
NaCl (100 mM) + CNT (100 μg/mL)	0.505 ± 0.276*	0.8 ± 0.447**	0.01274 ± 0.007*	0.00153 ± 0.0008*
NaCl (100 mM) + graphene (100 μg/mL)	0.465 ± 0.259*	0.875 ± 0.506*	0.00923 ± 0.005**	0.00105 ± 0.0006**

[a]Control plants were incubated on MS medium with and without supplements. *, $p < 0.05$ and **, $p < 0.01$ compared to control (untreated plants). One-way ANOVA was used for calculating treatment differences using post-hoc analysis via the Tukey test. ± represents standard error values.

Table 2. Total Number of Tomato Genes Affected by NaCl (100 mM), CNT (100 μg/mL), or Graphene (100 μg/mL) Added to MS Control and MS Medium Supplemented with NaCl

additions to the growth medium	total number of expressed genes	total number of DEGs compared to control		number of genes that fully or partially restored the level of expression as a result of the introduction of CBNs to medium supplemented with NaCl	
NaCl (100 mM)	22,555	3264	up: 1610 down: 1654	NA	
NaCl + CNT	22,703	3074	up: 1626 down: 1448	1639	up: 869 down: 770
NaCl + graphene	23,414	3151	up: 1701 down: 1450	1391	up: 749 down: 642
CNT	22,689	257	up: 216 down: 41	NA	
graphene	22,116	941	up: 575 down: 366	NA	
control (no treatment)	22,671	NA		NA	

graphene, or on MS medium containing NaCl (100 mM) in the presence of 100 μg/mL of graphene. Development of seedlings was observed for 21 days. From 100 tomato seeds incubated in the presence of NaCl, only three very short seedlings developed (Figure 2, Table 1). Growth of all tomato seedlings exposed to 100 mM NaCl fully stopped after 2 weeks of cultivation under salt stress. The addition of CNTs or graphene to the growth medium led to the development of tomato seedlings with significantly longer roots and shoots (Figure 2; Table 1). The positive influence of CBNs on plant growth and development is well documented and was described previously.[2,3,7] This study suggests that the addition of CNTs or graphene to a medium supplemented with NaCl may induce the development and growth of tomato seedlings in salty conditions (Figure 2). Tomato seedlings grown in media supplemented with salt and CBNs produced longer shoots and roots compared to seedlings grown on salty media without CBNs (Figure 2, Table 1).

To clarify how the whole tomato transcriptome will respond to salt stress in the absence and presence of CBNs (CNT, graphene), RNA-Seq was performed on tomato leaves collected from 21 day old plants exposed to NaCl, CNT, graphene, NaCl + CNT, and NaCl + graphene (Table 2). To identify the level of gene expression and the various degrees of expression between treatment groups, genes with adjusted p-values of <0.05 were considered as differentially expressed genes (DEGs). Bioinformatics analysis of RNA-Seq data revealed that all performed treatments significantly changed the total gene expression in tomato seedlings (Figure S1). Venn analysis identified the number of DEGs between control and seedlings treated with NaCl, NaCl + CNT, and NaCl + graphene (Figure S2). The degree of variation of DEG expression was visualized by volcano plots (Figure S3). To understand the biological significance of DEGs, Gene Ontology (GO) enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis were performed. GO analysis allowed for the annotation of genes to certain biological functions and KEGG helped to sort genes, according to the involvement of the biological pathway (Figures S4 and S5). The summary of the analysis of the total number of genes affected by performed treatments (NaCl, CNT, graphene, NaCl + CNT, NaCl + graphene) is shown in Table 2.

Overall, 3264 genes were differentially regulated in tomato seedlings in response to the application of 100 mM NaCl. 49.32% of NaCl-affected genes were upregulated and 50.67% of them were downregulated, compared to the control level. From this set of genes, the number of genes that fully or partially restored expression toward their level in control seedlings (untreated by NaCl) could be identified. Such restoration was achieved either by upregulation of genes suppressed by NaCl or by downregulation of genes activated by NaCl. The addition of CNT to salty medium (NaCl + CNT treatment) positively affected 1639 genes: 869 genes were upregulated, and 770 genes were downregulated compared to NaCl treatment (Table 2). Similarly, application of graphene resulted in full or partial restoration of expression of 1391 tomato genes affected by NaCl: 749 genes were upregulated,

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and 642 genes were downregulated. We confirmed data of RNA-Seq by analyzing the expression of selected genes using real-time PCR. Figure S6 represents amplification of the tomato *PR1* gene by real-time PCR in control seedlings and seedlings exposed to all treatments. Results of real-time PCR showed a trend that was observed in RNA-seq previously: suppression of *PR1* expression by NaCl and significant restoration of expression of a gene by application of CNT and graphene (Figure S6).

From all the DEGs affected by salt, 50.2 and 42.6% were fully or partially restored by application of CNTs and graphene, respectively (Table 2). NCBI BLAST and KEGG analysis were used to identify and annotate a wide range of restored genes, according to their function and involvement in specific biological processes (Tables S2 and S3). Tables S2A,C and S3A,C represent the genes that fully restored their level of expression in the presence of graphene or CNTs in response to salt stress. Overall, 119 and 48 annotated genes fully restored their level of expression toward control through upregulation and downregulation as a result of the application of graphene and CNTs, respectively (Tables S2A,C and S3A,C). During the analysis of partially restored genes, it was discovered that 1272 graphene-sensitive and 1592 CNT-sensitive genes in treated seedlings partially changed their level of expression toward the control seedlings. To further elucidate these expression changes, the ratio of change in the expression level was calculated for genes that reached 50% greater expression in the presence of CBNs compared to that of CBN-unexposed but salt-stressed seedlings (Tables S2B,D and S3B,D). After establishing this threshold, it was found that 45 annotated genes had at least a 50% shift in the expression level by either upregulation or downregulation as a result of the application of graphene, moving expression levels closer to that of the control. Similarly, 40 genes reached more than a 50% shift in the expression level toward control values after adding CNT to salty medium (Tables S2B,D and S3B,D). From all annotated 164 genes (upregulated and downregulated) that fully and partially restored their level of expression after adding graphene (Table S2A−D), 33.3% belonged to defense responses in plants, and from all annotated 88 genes (upregulated and downregulated) that fully and partially restored their level of expression after adding CNT (Table S3A−D), 26.1% are related to stress-responsive biological pathways. Stress-responsive genes can be classified into three major groups: (1) genes that encode products that directly protect plant cells against stresses such as heat-shock proteins (HSPs) or chaperones, LEA proteins, detoxification enzymes, and free-radical scavengers, (2) genes that are involved in signaling pathways and transcriptional factors, such as mitogen-activated protein kinases (MAPK), calcium-dependent protein kinases (CDPK), and (3) genes that are involved in water and ion uptake and transport such as aquaporins and ion transporters.[34] A number of genes from major groups of stress-related genes showed a trend to the restoration of expression affected by salt stress in response to CBN treatment (Tables S2 and S3). For example, aquaporins *TIP2-1* and *PIP2-6* restored their expression fully and partially in response to CNT (Table S3). The resulting modification of aquaporin expression was not surprising as this laboratory has produced previous evidence of upregulation in different aquaporin genes in various plant tissues, including the seeds of crops,[5] tomato leaves,[2] and seedlings of sorghum,[10] after the application of CNTs. It is known that salt can inhibit the expression of aquaporins, and the relationship between the expression levels of tomato aquaporins (PIPs) in response to salt stress has been noted recently by Jia et al.[35] The authors showed that the expression of PIPs decreased in roots and leaves in the first 24 h of exposure to salt, but plants were able to restore PIP expression after a recovery treatment in control nutrient solution.[35] Here, we demonstrate that CBNs can play a role of positive regulators of tomato aquaporin's expression suppressed by salt. CBNs can regulate the expression of transcriptional factors as well. As shown in Tables S2−S3, the expression levels of WRKY transcription factors (*WRKY 40* and *WRKY 31* genes) were regulated by CBNs under imposed salt stress. WRKY transcription factors are considered as key regulators of abiotic stress tolerance in plants.[36] *WRKY* transcription factors can bind to the W-box in the promoter of target genes, leading to activation or repression of downstream stress-responsive genes and regulate that response.[37] Hichri et al. demonstrated the role of *WRKY* transcription factors and salt tolerance in tomato using 35S::SlWRKY3 transgenic plants.[38] We observed that *DNAJ* gene-encoded protein associated with HSPs (DNAJ-like protein) restored the expression toward the control level in the presence of graphene or CNTs (Tables S2 and S3). DNAJ proteins are co-chaperones that work together with HSP70s to control protein homeostasis.[39] HSPs are major regulators of the abiotic stress response in plants.[40] In a study performed by Jungkunz et al., heat treatment of plants deficient in AtHsp70−15 dramatically increased mortality; this shows that AtHsp70−15 performed an important role in *Arabidopsis* plants against heat stress during normal growth.[41] We also observed that several genes such as pathogenesis-related protein 1 (*PR1* gene), protein kinase SOBIR1, inositol 2-dehydrogenase, and MLP-like protein 43 related to MAPK signaling, $InsP_3$ metabolism, and ABA signal pathways were fully or partially restored in salt-stressed seedlings treated with CNTs or graphene, closely resembling those of control seedlings (Tables S2 and S3). Since ABA and MAPK signaling pathways are major regulators for stress-related gene expression and adaptive physiological responses in plants,[42] the effect of CBNs on the overall plant stress response can be described as dramatic. Major changes in NaCl-affected gene expression caused by CBNs can explain the observed salt-tolerant phenotype of NaCl + CBN-exposed tomato seedlings (Figure 2, Table 1). Pandey et al. provided experimental evidence that CBNs can contribute to the salt-tolerant phenotype of NaCl-treated plants by interaction and uptake of free, positively charged ions.[10] Changes in transcriptome caused by CBNs and physical interaction of CBNs with harmful surroundings may play a role in the induction of stress-tolerant phenotype in CBN-exposed plants. To understand whether CBNs can contribute to tolerance to water-deficit stress by affecting the whole transcriptome, we performed a drought experiment involving rice plants.

Whole-Transcriptome Responses to Drought Stress in Rice Plants Treated with CBNs. While CBNs were previously shown to improve drought tolerance in plants such as *Catharanthus*, the effect that CBNs have on the drought-impacted plant transcriptome was still unknown.[9] In order to determine how CBNs improve drought tolerance, we applied CNTs and graphene to rice plants, performed an assessment of drought tolerance, and analyzed the transcriptome of stressed plants exposed to CBNs by RNA-seq. We found that the application of CNTs or graphene to rice plants resulted in the

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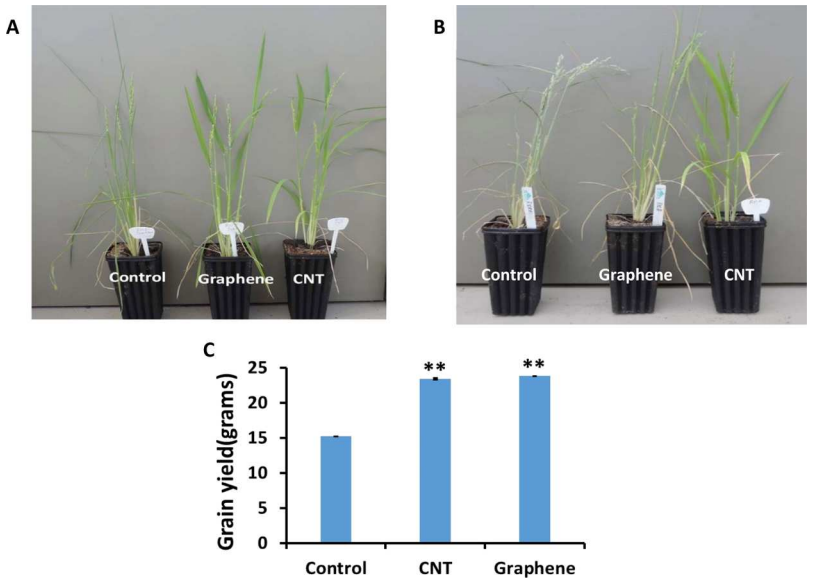

Figure 3. Phenotype and yield of rice plants exposed to CNT and graphene after water-deficit stress. The phenotype of rice plants exposed to CNT-(200 μg/mL) treated and graphene- (200 μg/mL) treated plants compared to the untreated plants during 25 days of water-deficit stress (A) and 31 days of water-deficit stress (B). Increase in the seed yield (C) in the CNT- and graphene-treated plants compared to the control plants after 41 days of water-deficit stress. Delivery of CBNs to soil mix was achieved by the addition of CNTs or graphene solution to the soil for 4 weeks. The final concentration of CNT and graphene was 80 mg per 400 g of soil mix. The experiments were repeated twice (n = 10 for each treatment and control and ** = p < 0.01).

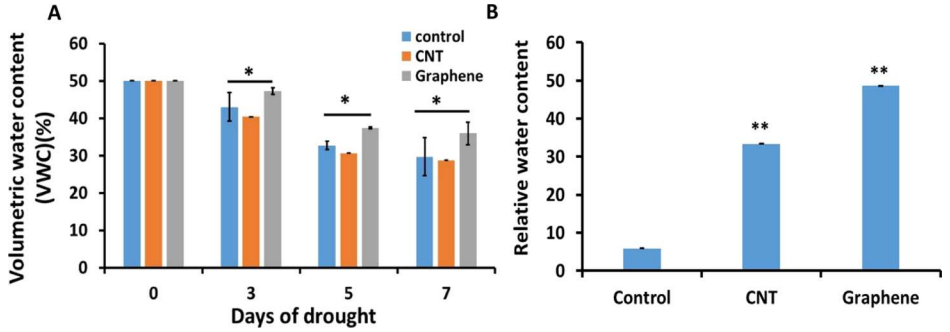

Figure 4. Effect of CBNs on the leaf RWC and soil moisture content (VWC) of rice plants cultivated in CNT- and graphene-supplemented soil after 7 days of water-deficit conditions. Soil moisture is expressed as the volumetric water content in pots used for cultivation of treated (CNT and graphene) and untreated rice plants during 0, 3, 5, and 7 days of water-deficit stress (A). Six plants were evaluated for each treatment. Increased RWC (B) in the leaves of CNT- and graphene-treated rice plants compared to the untreated plants after 7 days of water-deficit conditions. Six leaves were evaluated for each treatment (n = 6, * = p < 0.05, and ** = p < 0.01).

Table 3. Total Number of Rice Genes Impacted by Water Deficit in the Absence and Presence of CNT and Graphene[a]

treatment conditions	total number of expressed genes	total number of DEGs compared to control		number of genes that fully or partially restored the level of expression during drought stress after adding CBNs	
control (water-deficit conditions)	19,982	5472	up: 2695 down: 2777	NA	
CNT + water-deficit	20,403	1116	up: 587 down: 529	458	up: 267 down: 191
graphene + water-deficit	21,068	3353	up: 1946 down: 1407	1620	up: 760 down: 860
CNT + regular conditions	21,966	6279	up: 3188 down: 3091	NA	
graphene + regular conditions	24,958	6864	up: 4226 down: 2638	NA	
control (regular conditions)	24,105	NA		NA	

[a]Leaf samples were collected from 3 month old plants grown in regular conditions of watering or conditions of water-deficit (full withholding of water) in the presence or absence of CBNs (CNT, graphene).

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enhancement of drought stress tolerance. As shown in Figure 3A, the CNT- and graphene-treated plants stayed green and healthy even after 25 days of imposed water-deficit stress, whereas the control plants showed visible symptoms of wilting and leaf rolling on the 25th day (Figure 3A). Even though the graphene-treated plants began to show symptoms of wilting slowly on the 31st day of the stress experiment, the CNT-treated plants remained fresh and green (Figure 3B). Only on the 40th day, the CNT-treated plants showed signs of wilting and leaf rolling. The seed yield was higher in the CNT- and graphene-treated plants, showing an increase of 53.9% ($p < 0.01$) and 56.5% ($p < 0.01$), respectively, compared to the control plants (Figure 3C). The pots used for cultivation of the treated plants maintained more moisture than pots with the control plants during 7 days of cultivation without watering (Figure 4A). The RWC in the leaves of CNT- and graphene-treated plants was more than four times ($p < 0.01$) and more than seven times ($p < 0.01$) higher compared to RWC in leaves of the untreated rice plants, respectively (Figure 4B). We can state that the application of CBNs increased the plant's tolerance to water-deficit stress.

To further investigate the CBN effect on the total transcriptome of rice plants exposed to prolonged water-deficit stress, analysis of the rice plant transcriptome not exposed to water-deficit stress and those exposed to 25 days of water-deficit in the presence and absence of CBNs (CNT, graphene) was achieved with RNA Seq. Comparisons between treatments are presented as Venn analysis of DEGs (Figure S7), heat map (Figure S8), and volcano plots (Figure S9). Analysis of established data indicated that all treatments (drought, drought + CNT, drought + graphene, regular watering + CNT, and regular watering + graphene) significantly modified the total gene expression in rice. 5472 transcripts were differently regulated in rice plant leaves in response to water-deficit stress (Table 3). Addition of CNTs or graphene in the soil mix used for the cultivation of regularly watered plants affected 6279 and 6864 genes, respectively (Table 3).

As seen previously for other crop species exposed to salt stress (Figure 1; Table 2), the addition of CNT to the growth medium used for cultivation stress-affected plants both CBNs (CNTs and graphene) fully or partially restored expression of a significant portion of rice genes affected by drought. 458 and 1620 rice genes showed a trend to the restoration of gene expression affected by drought (Table 3) as a result of the application of CBNs to the soil mix. Identification of biological functions/pathways associated with genes with CBN-restored expression and annotation of selected genes was carried out by GO enrichment analysis, KEGG, and NCBI BLAST (Figures S10 and S11 and Tables S4 and S5). We refined the number of transcripts for discussion using a specific threshold levels for log2 fold change (250% for upregulation and 350% for downregulation in expression levels) compared to that of plants subjected to drought without the addition of CBNs (Tables S4 and S5). Application of graphene upregulated several genes, with 30 genes showing full restoration (Table S4A) and 17 showing partial restoration, approaching expression levels observed in control plants (Figure S4B). Treatment with graphene resulted in restoration through downregulation as well: 37 genes showed complete restored expression (Table S4C), and 40 genes showed partial restoration of expression (Table S4D). Application of CNTs in drought-stressed rice had similar results, as shown in Tables S5A,B, one gene was fully restored through upregulation, and 26 genes were partially restored through upregulation. Of the genes restored by downregulation, 17 genes were completely restored, and 11 genes were partially restored (Table S5C,D). We found that of the 55 restored genes (upregulated or downregulated) in the CNT-treated plants, 16.3% were major defense-response related (Table S5), and 30.6% of the 124 restored genes (upregulated/downregulated) in graphene-treated rice were involved in the defense response and plant innate immunity (Table S4). RNA-Seq results were validated with real-time RT-qPCR. Data for two rice genes (*OsPIP-1* and *OsSIK-1*) suppressed by drought and upregulated by CNT and graphene in stressed rice plants are shown in Figure S12.

Interestingly, many genes affected by CBNs in sorghum and tomato exposed to NaCl were also influenced by CBNs in rice plants grown under water-deficit stress. For example, expression of *WRKY 24* transcriptional factors were upregulated by CNT in rice (Table S5). Similarly, with the findings in sorghum (Figure 1), rice gene expression for *NAC*-domain-containing protein was positively influenced by the application of graphene (Table S4). Additionally, other genes for other transcriptional factors (*MYB 30*, *HY5*, *bHLH100*, and Zinc finger protein *ZAT10*) associated with plant response to abiotic stress showed fully or partially restored expression after drought when graphene was applied before the water-deficit experiment (Table S4). As seen in experiments with sorghum and tomato plants, genes related to major signaling or defense pathways were positively regulated by CBNs in rice as well. Examples of such genes are inositol-polyphosphate 4-phosphatase ($InsP_3$ metabolism), calcium-transporting ATPase 2, Ca-binding protein CML22, Ca-binding annexin D4 (calcium transport or calcium-binding), ABA transporter G family member 25, ABA-related stress ripening protein 3, ankyrin repeat-containing protein *NPR4* (ABA metabolism and signaling), protein *TIFY 11c*, protein *DMR6-LIKE OXYGENASE 1*, *PRB-2* protein, bifunctional nuclease 1, and ferritin 1 (plant defense response). Since changes in the expression of these identified genes can be linked with the level of abiotic tolerance of plants, these data may hint a relationship between the observed drought tolerance of CBN-treated rice plants (Figure 3) and the unique ability of tested CBNs affecting the stress-related plant transcriptome (Tables S4 and S5).

CONCLUSIONS

Our data confirmed the significant effect of CBNs on total plant gene expression. The influence of two types of CBNs (CNTs and graphene) on the total transcriptome of three agricultural plant species (sorghum, rice, and tomato) exposed to two types of abiotic stress (salt stress and water-deficit stress) was demonstrated using RNA-seq and real-time PCR. This novel data describe the unique ability of CBNs to restore expression (partially or fully) of a wide range of key stress-signaling genes affected by abiotic stress. The major families of genes that showed a trend to the restoration of expression toward the control level using treatment with CBNs were consistent across tested plant species and two types of imposed environmental stresses. CBNs positively influenced the expression of some MAPK signaling genes, ABA-signaling, genes, aquaporins, dehydrins, genes of HSPs/co-chaperones associated with HSPs, genes of $InsP_3$ signaling, RNA/protein processing transcripts, and genes involved in defense signaling and sugar metabolism in tested plant species exposed to drought and salt. It is possible that the effect of CBNs on the expression of stress-responsive genes was indirect and appeared

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through the effects of CBNs on other associated genes or pathways. Overall, the performed study can contribute to the clarification of the underlying biological mechanisms behind the positive and negative effects of nano-sized materials *in planta*. Based on the similarity of genes regulated in all tested plant species exposed to abiotic stresses, we can hypothesize that mechanisms of effects of different CBNs (CNTs and graphene) are the same in different crop species. However, understanding how and why CBNs can lead to significant changes in stress-related plant gene expression requires further detailed investigation.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsabm.1c00108.

Primers of selected genes for sorghum; annotated genes that fully or partially restored their expression in the presence of CBNs for tomato seedlings; annotated genes that fully or partially restored their expression in the presence of CBNs for rice plants; heat map of RNA-seq analysis of tomato; Venn analysis of tomato; volcano plots of DEGs of tomato; GO enrichment analysis of tomato; KEGG pathway analysis of tomato; relative transcript abundances of tomato PR1 gene; Venn analysis of rice plants; heat map of RNA-seq analysis of rice plants; volcano plots of DEGs of rice plants; GO enrichment analysis of rice plants; KEGG pathway analysis of rice plants; and relative transcript abundances of rice OsPIP1-1 and OsSIK-1 genes (PDF)

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Madannejad, R.; Shoaie, N.; Jahanpeyma, F.; Darvishi, M. H.; Azimzadeh, M.; Javadi, H. Toxicity of Carbon-Based Nanomaterials: Reviewing Recent Reports in Medical and Biological Systems. *Chem.-Biol. Interact.* **2019**, *307*, 206−222.

(2) Khodakovskaya, M. V.; de Silva, K.; Nedosekin, D. A.; Dervishi, E.; Biris, A. S.; Shashkov, E. V.; Galanzha, E. I.; Zharov, V. P. Complex Genetic, Photothermal, and Photoacoustic Analysis of Nanoparticle-Plant Interactions. *Proc. Natl. Acad. Sci. U.S.A.* **2011**, *108*, 1028−1033.

(3) Villagarcia, H.; Dervishi, E.; de Silva, K.; Biris, A. S.; Khodakovskaya, M. V. Bioresponse to Nanotubes: Surface Chemistry of Carbon Nanotubes Impacts the Growth and Expression of Water Channel Protein in Tomato Plants (Small 15/2012). *Small* **2012**, *8*, 2327.

(4) Khodakovskaya, M. V.; Kim, B.-S.; Kim, J. N.; Alimohammadi, M.; Dervishi, E.; Mustafa, T.; Cernigla, C. E. Carbon Nanotubes as Plant Growth Regulators: Effects on Tomato Growth, Reproductive System, and Soil Microbial Community. *Small* **2013**, *9*, 115−123.

(5) Lahiani, M. H.; Dervishi, E.; Chen, J.; Nima, Z.; Gaume, A.; Biris, A. S.; Khodakovskaya, M. V. Impact of Carbon Nanotube Exposure to Seeds of Valuable Crops. *ACS Appl. Mater. Interfaces* **2013**, *5*, 7965−7973.

(6) Lahiani, M. H.; Chen, J.; Irin, F.; Puretzky, A. A.; Green, M. J.; Khodakovskaya, M. V. Interaction of Carbon Nanohorns with Plants: Uptake and Biological Effects. *Carbon* **2015**, *81*, 607−619.

(7) Lahiani, M. H.; Dervishi, E.; Ivanov, I.; Chen, J.; Khodakovskaya, M. Comparative Study of Plant Responses to Carbon-Based Nanomaterials with Different Morphologies. *Nanotechnology* **2016**, *27*, 265102.

(8) Lahiani, M. H.; Gokulan, K.; Williams, K.; Khodakovskaya, M. V.; Khare, S. Graphene and Carbon Nanotubes Activate Different Cell Surface Receptors on Macrophages before and after Deactivation of Endotoxins. *J. Appl. Toxicol.* **2017**, *37*, 1305−1316.

(9) Pandey, K.; Anas, M.; Hicks, V. K.; Green, M. J.; Khodakovskaya, M. V. Improvement of Commercially Valuable Traits of Industrial Crops by Application of Carbon-Based Nanomaterials. *Sci. Rep.* **2019**, *9*, 19358.

(10) Pandey, K.; Lahiani, M. H.; Hicks, V. K.; Hudson, M. K.; Green, M. J.; Khodakovskaya, M. Effects of Carbon-Based Nanomaterials on Seed Germination, Biomass Accumulation and Salt Stress Response of Bioenergy Crops. *PLoS One* **2018**, *13*, No. e0202274.

(11) Paramo, L. A.; Feregrino-Pérez, A. A.; Guevara, R.; Mendoza, S.; Esquivel, K. Nanoparticles in Agroindustry: Applications, Toxicity, Challenges, and Trends. *Nanomaterials* **2020**, *10*, 1654.

(12) Barrs, H.; Weatherley, P. A Re-Examination of the Relative Turgidity Technique for Estimating Water Deficits in Leaves. *Aust. J. Biol. Sci.* **1962**, *15*, 413−428.

(13) Klein, P. E.; Klein, R. R.; Cartinhour, S. W.; Ulanch, P. E.; Dong, J.; Obert, J. A.; Morishige, D. T.; Schlueter, S. D.; Childs, K. L.; Ale, M.; Mullet, J. E. A High-Throughput AFLP-Based Method for Constructing Integrated Genetic and Physical Maps: Progress toward a Sorghum Genome Map. *Genome Res.* **2000**, *10*, 789−807.

(14) Buchanan, C. D.; Lim, S.; Salzman, R. A.; Kagiampakis, I.; Morishige, D. T.; Weers, B. D.; Klein, R. R.; Pratt, L. H.; Cordonnier-Pratt, M.-M.; Klein, P. E.; Mullet, J. E. Sorghum Bicolor's Transcriptome Response to Dehydration, High Salinity and ABA. *Plant Mol. Biol.* **2005**, *58*, 699−720.

(15) Rorat, T. Plant Dehydrins - Tissue Location, Structure and Function. *Cell. Mol. Biol. Lett.* **2006**, *11*, 536−556.

(16) Hanin, M.; Brini, F.; Ebel, C.; Toda, Y.; Takeda, S.; Masmoudi, K. Plant Dehydrins and Stress Tolerance. *Plant Signaling Behav.* **2011**, *6*, 1503−1509.

(17) Rinne, P. x. i. L. H.; Kaikuranta, P. x. i. L. M.; Van Der Plas, L. H. W.; Van Der Schoot, C. Dehydrins in Cold-Acclimated Apices of Birch (Betula Pubescens Ehrh.): Production, Localization and Potential Role in Rescuing Enzyme Function during Dehydration. *Planta* **1999**, *209*, 377−388.
(18) Koag, M.-C.; Fenton, R. D.; Wilkens, S.; Close, T. J. The Binding of Maize DHN1 to Lipid Vesicles. Gain of Structure and Lipid Specificity. *Plant Physiol.* **2003**, *131*, 309−316.
(19) Yu, Z.; Wang, X.; Zhang, L. Structural and Functional Dynamics of Dehydrins: A Plant Protector Protein under Abiotic Stress. *Int. J. Mol. Sci.* **2018**, *19* (11), 3420.
(20) Hara, M.; Fujinaga, M.; Kuboi, T. Radical Scavenging Activity and Oxidative Modification of Citrus Dehydrin. *Plant Physiol. Biochem.* **2004**, *42*, 657−662.
(21) Saavedra, L.; Svensson, J.; Carballo, V.; Izmendi, D.; Welin, B.; Vidal, S. A Dehydrin Gene in Physcomitrella Patens Is Required for Salt and Osmotic Stress Tolerance. *Plant J.* **2006**, *45*, 237−249.
(22) Zhu, J.-K. Cell Signaling under Salt, Water and Cold Stresses. *Curr. Opin. Plant Biol.* **2001**, *4*, 401−406.
(23) Himmelbach, A.; Yang, Y.; Grill, E. Relay and Control of Abscisic Acid Signaling. *Curr. Opin. Plant Biol.* **2003**, *6*, 470−479.
(24) Shinozaki, K.; Yamaguchi-Shinozaki, K.; Seki, M. Regulatory Network of Gene Expression in the Drought and Cold Stress Responses. *Curr. Opin. Plant Biol.* **2003**, *6*, 410−417.
(25) Riechmann, J. L.; J Heard, G. M.; Reuber, L.; Jiang, C.-Z.; Keddie, J.; Adam, L.; Pineda, O.; Ratcliffe, O. J.; Samaha, R. R.; Creelman, R.; Pilgrim, M.; Broun, P.; Zhang, J. Z.; Ghandehari, D.; Sherman, B. K.; Yu, G.-L. Arabidopsis Transcription Factors: Genome-Wide Comparative Analysis Among Eukaryotes. *Science* **2000**, *290*, 2105−2110.
(26) Takada, S.; Hibara, K.; Ishida, T.; Tasaka, M. The CUP-SHAPED COTYLEDON1 Gene of Arabidopsis Regulates Shoot Apical Meristem Formation. *Development* **2001**, *128*, 1127−1135.
(27) Sablowski, R. W. M.; Meyerowitz, E. M. A Homolog of NO APICAL MERISTEM Is an Immediate Target of the Floral Homeotic Genes APETALA3/PISTILLATA. *Cell* **1998**, *92*, 93−103.
(28) Zhong, R.; Lee, C.; Ye, Z.-H. Global Analysis of Direct Targets of Secondary Wall NAC Master Switches in Arabidopsis. *Mol. Plant* **2010**, *3*, 1087−1103.
(29) Breeze, E.; Harrison, E.; McHattie, S.; Hughes, L.; Hickman, R.; Hill, C.; Kiddle, S.; Kim, Y.-s.; Penfold, C. A.; Jenkins, D.; Zhang, C.; Morris, K.; Jenner, C.; Jackson, S.; Thomas, B.; Tabrett, A.; Legaie, R.; Moore, J. D.; Wild, D. L.; Ott, S.; Rand, D.; Beynon, J.; Denby, K.; Mead, A.; Buchanan-Wollaston, V. High-Resolution Temporal Profiling of Transcripts during Arabidopsis Leaf Senescence Reveals a Distinct Chronology of Processes and Regulation. *Plant Cell* **2011**, *23*, 873−894.
(30) Olsen, A. N.; Ernst, H. A.; Leggio, L. L.; Skriver, K. NAC Transcription Factors: Structurally Distinct, Functionally Diverse. *Trends Plant Sci.* **2005**, *10*, 79−87.
(31) Zheng, X.; Chen, B.; Lu, G.; Han, B. Overexpression of a NAC Transcription Factor Enhances Rice Drought and Salt Tolerance. *Biochem. Biophys. Res. Commun.* **2009**, *379*, 985−989.
(32) Wang, W.; Vinocur, B.; Shoseyov, O.; Altman, A. Role of Plant Heat-Shock Proteins and Molecular Chaperones in the Abiotic Stress Response. *Trends Plant Sci.* **2004**, *9*, 244−252.
(33) Afzal, Z.; Howton, T.; Sun, Y.; Mukhtar, M. The Roles of Aquaporins in Plant Stress Responses. *J. Dev. Biol.* **2016**, *4*, 9.
(34) Shanker, A.; Venkateswarlu, B. *Abiotic Stress in Plants: Mechanisms and Adaptations*; BoD—Books on Demand, 2011.
(35) Jia, J.; Liang, Y.; Gou, T.; Hu, Y.; Zhu, Y.; Huo, H.; Guo, J.; Gong, H. The Expression Response of Plasma Membrane Aquaporins to Salt Stress in Tomato Plants. *Environ. Exp. Bot.* **2020**, *178*, 104190.
(36) Phukan, U. J.; Jeena, G. S.; Shukla, R. K. WRKY Transcription Factors: Molecular Regulation and Stress Responses in Plants. *Front. Plant Sci.* **2016**, *7*, 760.
(37) Jiang, J.; Ma, S.; Ye, N.; Jiang, M.; Cao, J.; Zhang, J. WRKY Transcription Factors in Plant Responses to Stresses. *J. Integr. Plant Biol.* **2017**, *59*, 86−101.
(38) Hichri, I.; Muhovski, Y.; Žižková, E.; Dobrev, P. I.; Gharbi, E.; Franco-Zorrilla, J. M.; Lopez-Vidriero, I.; Solano, R.; Clippe, A.; Errachid, A.; Motyka, V.; Lutts, S. The Solanum Lycopersicum WRKY3 Transcription Factor SlWRKY3 Is Involved in Salt Stress Tolerance in Tomato. *Front. Plant Sci.* **2017**, *8*. DOI: DOI: 10.3389/fpls.2017.01343.
(39) Cheetham, M. E.; Caplan, A. J. Structure, Function and Evolution of DnaJ: Conservation and Adaptation of Chaperone Function. *Cell Stress Chaperones* **1998**, *3*, 28−36.
(40) Banerjee, A.; Roychoudhury, A. Chapter 19—Small Heat Shock Proteins: Structural Assembly and Functional Responses Against Heat Stress in Plants. In *Plant Metabolites and Regulation under Environmental Stress*; Ahmad, P., Ahanger, M. A., Singh, V. P., Tripathi, D. K., Alam, P., Alyemeni, M. N., Eds.; Academic Press, 2018; pp 367−376.
(41) Jungkunz, I.; Link, K.; Vogel, F.; Voll, L. M.; Sonnewald, S.; Sonnewald, U. AtHsp70-15-Deficient Arabidopsis Plants Are Characterized by Reduced Growth, a Constitutive Cytosolic Protein Response and Enhanced Resistance to TuMV. *Plant J.* **2011**, *66*, 983−995.
(42) Danquah, A.; de Zelicourt, A.; Colcombet, J.; Hirt, H. The Role of ABA and MAPK Signaling Pathways in Plant Abiotic Stress Responses. *Biotechnol. Adv.* **2014**, *32*, 40−52.

https://doi.org/10.1021/acsabm.1c00108
ACS Appl. Bio Mater. XXXX, XXX, XXX−XXX