Carbon Nanotubes Induce Growth Enhancement of Tobacco Cells

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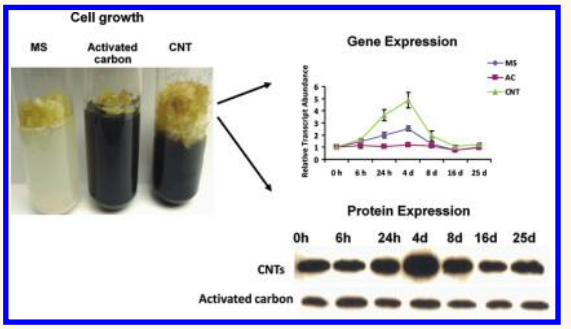
ABSTRACT



Carbon nanotubes have shown promise as regulators of seed germination and plant growth. Here, we demonstrate that multiwalled carbon nanotubes (MWCNTs) have the ability to enhance the growth of tobacco cell culture (55—64% increase over control) in a wide range of concentrations (5—500 μg/mL). Activated carbon (AC) stimulated cell growth (16% increase) only at low concentrations (5 μg/mL) while dramatically inhibited the cellular growth at higher concentrations (100—500 μg/mL). We found a correlation between the activation of cells growth exposed to MWCNTs and the upregulation of genes involved in cell division/cell wall formation and water transport. The expression of the tobacco aquaporin (*NtPIP1*) gene, as well as production of the *NtPIP1* protein, significantly increased in cells exposed to MWCNTs compared to control cells or those exposed to AC. The expression of marker genes for cell division (*CycB*) and cell wall extension (*NtLRX1*) was also up-regulated in cells exposed to MWCNTs compared to control cells or those exposed to activated carbon only.

KEYWORDS: carbon nanotubes · cell division · gene expression · aquaporin protein · Raman spectroscopy

Novel developments in the area of nanotechnology have provided advanced knowledge and technological platforms with applications in a variety of scientific areas, ranging from medicine, aerospace, electronics, and sensing to defense industries.[1—3] Lately, given the need to understand the interaction between engineered nanomaterials and various biological systems, a significant research interest has developed around the use of nanotechnology-based approaches for agricultural and food systems.[4] The unique properties of nanosized materials (small size, high biochemical reactivity, ability to penetrate cells, and swift distribution inside organisms) make them an attractive tool for crop management techniques. In this respect, it has been documented that nanoparticles can be beneficial for the delivery of biological molecules to plant cells[5—8] or to improve herbicide delivery.[9] Specific types of nanoparticles in low doses have not been found harmful to plants but instead are capable of activating specific physiological processes. For example, TiO_2 nanoparticles (0.25—4%) are able to promote photosynthesis and nitrogen metabolism in spinach and therefore improve the growth of the plants.[10,11] We have demonstrated recently that multiwalled carbon nanotubes (MWCNTs) at relatively low doses (10—40 μg/mL) can penetrate thick seed coats, stimulate germination, and activate enhanced growth in tomato plants.[12,13] All of these developments have the potential to transform agricultural practices in the near future and to provide solutions to some of the most serious problems related to plant growth and development.

Nevertheless, future perspectives on nanobiotechnological approaches for the regulation of plant productivity will depend on a thorough understanding of the molecular mechanisms responsible for the activation of seed germination and plant growth in the presence of complex engineered nanomaterials. We demonstrated earlier that MWCNTs can be taken up by tomato plants and affect their total gene expression.[13] For example, the expression of tomato aquaporin (water channel gene) and a number of other genes related to plant responses to environmental stress were found to be up-regulated in tomato seedlings by exposure to MWCNTs but not by exposure to activated carbon (AC), alone. To further understand the biological mechanisms that control the complex influence of carbon nanotubes on plants, the interactions between these tubular

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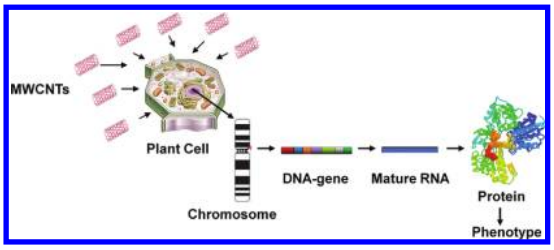

Figure 1. Schematic representation of the complex effects that multiwalled carbon nanotubes (MWCNTs) can induce at the cellular and molecular levels in living organisms. Interaction of plant cells with MWCNTs can lead to significant changes at the molecular level and affect the expression of specific genes and the production of proteins responsible for a particular phenotype.

nanostructures and plants need to be investigated at all levels of plant organization, including the cell, transcriptome, and proteome (Figure 1). To achieve this goal, a classical strategy can be applied. First, on the basis of phenotypical traits of the biological organism exposed to nanomaterials, the involvement of nanomaterials in specific cellular, developmental, signaling, or biosynthetic processes and pathways can be hypothesized. Next, key genes involved in such processes need to be identified as markers of selected biological processes. The expression of marker genes has to be studied in organisms exposed and unexposed to nanomaterials. As the next step, the expression level of the products of marker genes (proteins) has to be monitored in tested organisms. This strategy will shed light on the molecular mechanisms of positive or negative (toxic) effects of nanomaterials observed in any biological system exposed to nanosized materials.

This study is the first report, to our best knowledge, that highlights the positive effects that carbon nanomaterials have on plant cell division. Here, we demonstrate for the very first time, to our best knowledge, that the growth of tobacco cell culture (callus) can be affected and highly enhanced by the introduction of multiwalled carbon nanotubes in the growth medium. This effect was in direct correlation with the overexpression of genes-markers for cell division/extension in tobacco cells exposed to MWCNTs. Additionally, analysis of aquaporin gene and protein expression in control and MWCNT-exposed tobacco cells supported a previously formulated hypothesis[13] about the possible regulation of plant water channels (aquaporins) by the exposure to carbon nanotubes. Our findings represent a significant leap in the understanding of the impact that nanomaterials have on plants, down to the individual cell level, and contribute to clarification of the molecular mechanisms responsible for the nanotubes' positive impact on plant growth and development.

RESULTS AND DISCUSSION

To further understand the effects induced by carbon nanotubes on plant cell division/cell growth, we exposed cultures of undifferentiated tobacco cells (callus culture) grown on MS medium to a wide range of concentrations of multiwalled carbon nanotubes (MWCNTs) or activated carbon (AC), as the positive control.

Detection of MWCNTs in Tobacco Cells Grown on Medium Supplemented with MWCNTs. The interaction between MWCNTs and the tobacco cells grown on MS medium supplemented with MWCNTs was probed with Raman spectroscopy and additionally by transmission electron microscopy (TEM), as shown in Figure 2. The nanotubes' specific G band intensity (the most intense) was analyzed when the samples were exposed to a 633 nm laser excitation. The G band corresponds to the sp^2-stretching modes both in ring and chains for the graphitic structures and represents the E_{2g} mode at the center of the Brillouin zone.[14,15] The presence and relative intensity of the G band (1581 cm^{-1}) was associated with the existence of the MWCNTs[16] in the cell samples, as shown in Figure 2C. The Raman analysis did not indicate any similar peak in the spectra of the control samples not exposed to MWCNTs. This experimental finding is in good correlation with data that Liu *et al.* have presented previously and who demonstrated by confocal microscopy that carbon nanotubes have the ability to penetrate the walls of the tobacco cells.[7] It should be mentioned that most of the spectroscopic analytical techniques, such as Raman scattering, lack the ability to very accurately quantify/comparatively assess the amount of nanotubes present inside the cells, but rather can be used to prove the presence of nanotubes in the samples. To additionally show the uptake of MWCNTs by the tobacco cells, we performed TEM analysis for cells grown on agar medium with and without MWCNTs. TEM imaging clearly showed the presence of clustered nanotubes inside tobacco cells grown on the medium supplemented with 100 μg/mL of MWCNTs (Figure 2D). It is interesting that MWCNTs were detected inside the cells that were collected from the upper part of the callus biomass, which did not have any direct contact with the medium. During many rounds of cell division, cell biomass increased significantly. As a result, the cells used for the TEM transfer were most probably never directly exposed to the MWCNTs present in the medium. Therefore, as the cells divided, they move the nanotubes from one generation to the next. Furthermore, the presence of MWCNTs even in the upper cells indicates that the MWCNTs were uptaken in a significant amount by the initial cells population that had been in contact with the MWCNTs-supplemented medium. On the basis of these findings, it is expected that the interactions between carbon nanotubes and tobacco cells and the uptake of MWCNTs by cells have the potential to induce significant responses at the cellular and genetic levels.

Activation of Growth of Tobacco Cell Culture in the Presence of MWCNTs. To understand how different concentrations of MWCNTs would affect cell division in callus

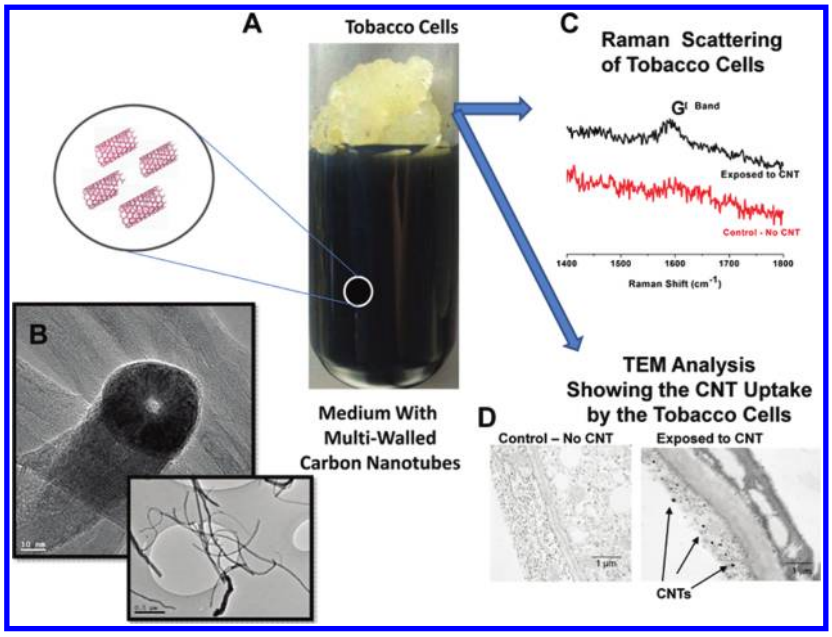

Figure 2. Detection of MWCNTs in tobacco cells grown on medium supplemented with MWCNTs. (A) Growth of tobacco cells on medium supplemented with 100 μg/mL of MWCNTs. (B) Representative transmission electron microscopy (TEM) image of the nanotubes used in this study. Nanotubes had an average diameter of 20 nm and lengths ranging from 500 nm to 1 μm. (C) Raman analysis of the cells exposed to the MWCNTs indicating that the tobacco plant cells uptake the nanotubes (based on the nanotube-specific 1581 cm^{-1}-G band). (D) TEM images of the tobacco cells exposed and unexposed to MWCNTs.

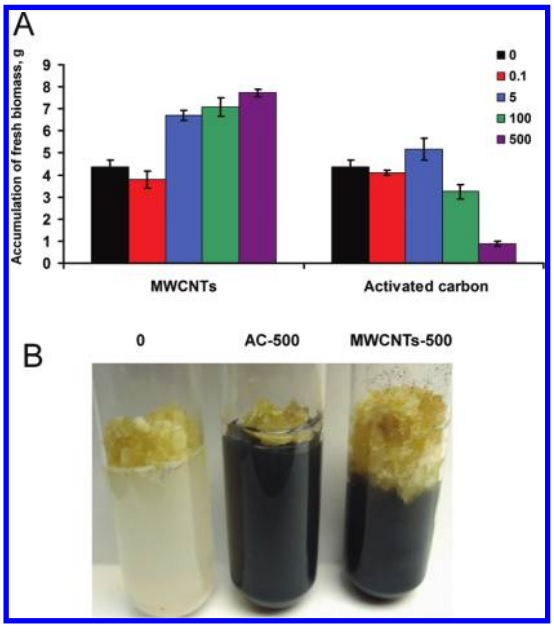

Figure 3. Effect of multiwalled carbon nanotubes (MWCNTs) and activated carbon on growth of tobacco cell culture. (A) Biomass accumulation of culture of tobacco cells grown on regular MS medium, MS medium supplemented with activated carbon (0.1, 5, 100, and 500 μg/mL), and MS medium supplemented with MWCNTs (0.1, 5, 100, and 500 μg/mL). (B) Differences in growth of control cells (0) and cells exposed to activated carbon (AC) or multiwalled carbon nanotubes (MWCNTs) in highest tested dose (500 μg/mL). Equal amount of biological material (300 mg) was used for all experimental conditions and all replicates. Each experimental condition was replicated 10 times (10 tubes were used for each concentration of carbon nanotubes, activated carbon, or control medium). The entire experiment was repeated twice. Thus, vertical bars indicate $\pm$SE (n = 20).

culture, the total accumulation of cell biomass (fresh weight and dry weight) was measured after 1 month of cell incubation with and without MWCNTs or with activated carbon. To standardize the weight-measuring experiments among the various experimental replicates (20 for each treatment), we used tubes specifically designed for plant tissue culture, as previously shown in our studies.[17,18] As presented in Figure 3A, both MWCNTs and AC affected tobacco cell growth. MWCNTs enhanced cell growth by 55% to 64% for the concentration range of 5−500 μg/mL. A more modest activation of cell growth (16% increase) was observed for the cells exposed to AC at low concentrations (5 μg/mL), while a significant decrease in cell growth was observed for high doses (100, 500 μg/mL). The average dry weight of biomasses accumulated under each experimental condition was measured as well. It was found that the dry weight of calluses was in direct correspondence with the fresh weight and represented 9% of the total fresh weight in all of the experimental conditions that were investigated (Supporting Information, Figure S1). Thus, the addition of MWCNTs to the medium resulted in an increase in both the fresh and dry weight of the calluses. This observation is an indication that an increase in cell growth is associated with the activation of cell division and not with an increase in cell volume through the enhancement of water uptake. The modest (16%) activation of growth in cells exposed to AC (5 μg/mL) could be explained by the previously described ability of activated carbon to adsorb substances presumed to be deleterious or inhibitory to callus growth during long-term cultivation.[19]

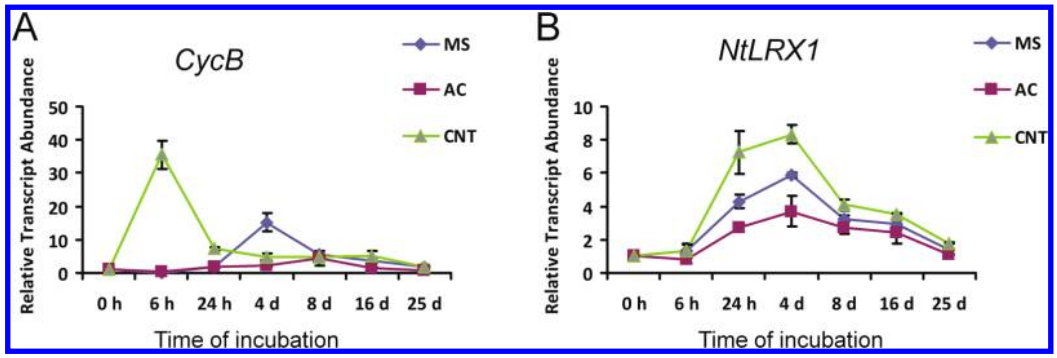

Figure 4. Relative transcript abundance of *CycB* (A) and *NtLRX1* (B) genes in tobacco cells cultured on standard medium (MS), MS medium supplemented with 100 μg/mL of MWCNTs (CNT), or supplemented with 100 μg/mL of activated carbon (AC). Expression of genes was analyzed by real-time PCR. Results are shown as the average of three independent biological replicates. Relative expression levels were normalized to an internal standard (actin) for each treatment. Bars represent the standard error (SE).

The inhibition of callus growth in medium supplemented with AC in higher doses (100 and 500 μg/mL) could be associated with the non-selective ability of AC to adsorb substances from the MS growth medium, including plant growth regulators, vitamins, iron chelate, and Zn.[20]

It is important to emphasize that MWCNTs in high concentration (500 μg/mL) were not found to be toxic to cells, but were instead able to stimulate cell growth (Figure 3B). Our findings have highlighted positive effects of multiwalled carbon nanotubes on tobacco cells, although this topic is still under intense scientific investigation, since several studies have reported contradictory results.[21,22] Earlier, various aspects describing the toxicity of multiwalled carbon nanotube agglomerates were reported for *Arabidopsis*[21] and rice[22] suspension cell cultures. One possible explanation for such results is related to the exact plant systems used for the investigations, as well as the conditions under which the nanotubes were delivered and their co-chemical characteristics. If the nanostructures agglomerate, their behavior could differ drastically from that observed when they are individually dispersed. Such contradictions in the cells' physiological responses to nanostructural materials may be further explained by the differences in the specific characteristics of the nanotubes used during the experiment (size, level of agglomeration, and distribution in growth medium), the duration of the incubation of cells with nanomaterials, the concentrations of carbon nanotubes, and the type of medium used for cultivation (solid or liquid), as well as the type and age of the plant material. Recently, we demonstrated the need for a thorough understanding of the complex bioactivity of each component of a nanoscaled system that is introduced to plant models, since each of these components could induce its own individual toxicity.[23] Particularly, in our case, the attachment of quantum dots (QD) to carbon nanotubes completely reversed the positive physiological responses that were recorded for the tomato plants exposed to carbon nanotubes only. Tomato plants grown on medium supplemented with QD-CNT aggregates exhibited symptoms of early leaf senescence and inhibition of root growth, which were not observed for the plants exposed to MWCNTs only. Moreover, we have clearly shown that even the morphology/shape of the graphitic nanomaterials (single-walled or multiwalled carbon nanotubes and graphene layers) can induce different physiological responses in tomato plants.[13]

Carbon Nanotubes Can Affect the Expression of Genes Involved in Cell Division and Extension. On the basis of these observations (Figure 3), we suggest the existence of different molecular mechanisms for cell growth activation by the nanosized MWCNTs and by AC. To test this hypothesis, we monitored the expression of genes essential for cell-wall assembly/cell growth, such as extensin (*NtLRX1*), and for the regulation of cell cycle progression, *CycB,* in tobacco cells grown on medium supplemented with 100 μg/mL of MWCNTs or AC, as well as on regular MS medium (control). Using real-time PCR analysis, we found that both genes exhibited the highest level of expression when the cells were treated with MWCNTs (Figure 4 A,B). The expression of the *CycB* gene was significantly elevated in MWCNT-treated tobacco cells, increasing by 35-fold after only 6 h of cell incubation. A 15-fold increase in *CycB* expression was detected on the fourth day of incubation in control (untreated) cells, whereas the lowest expression of *CycB* was found in cells treated with activated carbon (Figure 4A). Thus, the transcription of the cell cycle regulator *CycB* can be significantly and rapidly induced by MWCNTs in tobacco cells. Earlier, Schnittger *et al.* experimentally proved that ectopic *CycB1;2* expression is important for plant cell division and demonstrated that it can induce nuclear divisions and is sufficient to switch between endoreduplication and mitosis in *Arabidopsis* trichomes.[24] The expression analysis of the tobacco *CycB* gene (Figure 4A) is correlated with the observed enhanced growth of the cells exposed to MWCNTs (100 μg/mL) as compared to those exposed to AC (100 μg/mL) or the control samples (Figure 3).

We also monitored the expression of the *NtLRX1* gene (extensin1) which earlier studies have indicated

plays a key role in cell wall reinforcement during plant development and in response to external signals.[25,26] We recorded significant changes in the transcript abundance of the *NtLRX1* gene between the control, MWCNTs-exposed, and AC-exposed cells, during up to 25 days of incubation. The highest expression of *NtLRX1* was detected in cells incubated on medium supplemented with MWCNTs (100 μg/mL) between 1 and 4 days of exposure (Figure 4B). The *NtLRX1* expression was lower for the cells exposed to AC (100 μg/mL) and in control cells (2.5 fold and 1.5 fold, respectively). There is experimental evidence that plant extensins are synthesized in cells in response to physical damage or environmental stress conditions including wounding.[27,28] Our data suggest that nanosized carbon (MWCNTs) can be sensed by cells in a manner similar to an environmental stress. Therefore, it is possible that the overexpression of *NtLRX1* (extensin 1) has contributed to the observed enhancement in tobacco cell growth by MWCNTs. Previously, Bucher *et al*. demonstrated a direct correlation between the expression of *LeExt1* gene encoding tomato extensin-like protein and cellular tip growth.[28]

Additionally, we monitored the expression of both tested genes (*NtLRX1* and *CycB*) in cells grown on medium supplemented with very small amounts of MWCNTs or AC (5 μg/mL). We did not find any differences between the expression of both genes in control cells and cells exposed to a low amount of AC. However, expression of *NtLRX1* and *CycB* was activated in cells exposed to a small amount of MWCNTs compared with control and AC-exposed cells (Supporting Information, Figure S2). This observation supported our suggestion that molecular mechanisms for cell growth activation by the nanosized MWCNTs and by AC are indeed different.

Carbon Nanotubes Can Regulate Tobacco Water Channels (Aquaporins). On the basis of previous findings, we suggest that carbon nanotubes have multifaceted effects on plant transcriptome and can affect the expression of a number of genes that are essential for cellular functions. This assumption was further proved by analyzing the effect of MWCNTs on the expression of the water channel gene (*NtPIP1*) and the production of the corresponding *NtPIP1* protein (Figure 5) in tobacco cells. Significant up-regulation of the *NtPIP1* gene was detected by real-time PCR analysis in cells exposed to MWCNTs (100 μg/mL), from 24 h to 4 days of incubation, whereas the level of *NtPIP1* expression did not change for the cells grown on medium supplemented with AC (100 μg/mL). Additionally, the production of *NtPIP*1 protein during 25 days of cell incubation was monitored using Western blot analysis (Figure 5B). Consistent with gene expression data (Figure 5A), the production of *NtPIP1* protein reached a maximum on the fourth day of exposure to MWCNTs (100 μg/mL) and was higher than in control cells or in those treated with AC (100 μg/mL). Equal loading of the protein samples was assessed using antibodies raised against actin

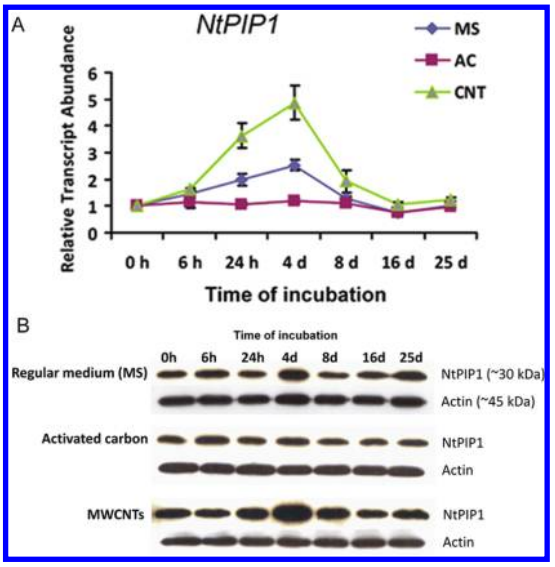

Figure 5. Analysis of the expression of tobacco aquaporin gene (*NtPIP1*) by real-time PCR (A) and of NtPIP1 protein by Western blot (B) during 25 days of incubation of cells grown on standard MS medium (MS), MS medium supplemented with 100 μg/mL of MWCNTs (CNT), or supplemented with 100 μg/mL of activated carbon (AC). Results of real-time PCR are shown as the average of three independent biological replicates. Actin was chosen as an internal control based on equal amplification efficiencies of actin and analyzed genes. Data for amplification efficiencies of actin and aquaporin are shown in Supporting Information, Figure S2. The relative expression levels were normalized to an internal standard (actin) for each treatment. Bars represent the standard error (SE). Western blot was repeated four times. Equal loading of the protein was assessed using anti-actin primary and anti-mouse IgG HRP secondary antibodies.

protein as shown in Figure 5B. There are many reports indicating that water channel proteins (aquaporins) are central components in plant-water relations and are crucial for root water uptake, seed germination, cell elongation, reproduction, and photosynthesis.[29,30] It has been shown that overexpression of *Arabidopsis* plasma membrane water channel gene (*PIP1b*) in tobacco plants leads to an increase in plant growth rate, transpiration rate, stomatal density, and photosynthetic efficiency.[31] The authors concluded that symplastic water transport *via* water channels represents a limiting factor for plant growth. Similarly, Sade *et al*. demonstrated that the constitutive expression of the *SLTIP2;2* gene (tomato aquaporin) resulted in an increase in the osmotic water permeability of the cells and that transgenic plants transpired more and for longer periods under drought stress compared to control plants.[32] *SLTIP2;2* expressing plants showed an increase in fruit yield, harvest index, and plant biomass by comparison with control tomato plants. It was shown also that the overexpression of aquaporin genes (*OsPIP1;3*) in seed embryos of rice promoted seed germination under water-stress conditions.[33] Our data provide the first evidence that carbon nanotubes are able to enhance the production of water channel protein, which may regulate cell growth.

Our discovery related to the significant effects that the MWCNTs have on plant gene expression raises important questions about the possible mechanisms that govern these effects. Previously, we have noted that many genes activated or inhibited by MWCNTs in tomato plants are involved in plant stress-signal transduction and can be regulated by specific environmental stress.[13] Taking into account the ability of MWCNTs to easily penetrate plant cell walls, we hypothesized that plants can sense carbon nanotubes as stress factors similar to pathogen attacks. We made such an assumption based on our experimental observations that carbon nanotubes can activate the same genes, as well as similar signaling pathways and cascades, that are normally activated in response to pathogen attacks.[13] However, we presented here new experimental data that clearly show that MWCNTs can also regulate the expression of genes that are involved in cell division/extension. These findings further highlight the fact that the effects that MWCNTs have on plant gene expression are rather complex and require further investigation. These comprehensive studies focused on an understanding of how plant cells sense and recognize nanosized materials at the molecular level. Therefore, a thorough comprehension of which signaling pathways are affected by nanomaterials could result in a better understanding of the effects of MWCNTs on plant transcriptome. Such findings could elucidate the impact that engineered nanomaterials have on plant biology and could open a new research area at the interface between nanomaterials, plant biology, disease control and treatment, and possibly the use of nanoscale agents for increase of drought tolerance.

CONCLUSION

These results suggest that carbon nanotubes can regulate cell division and plant growth by an unique molecular mechanism that is related to the activation of water channels (aquaporins) and major gene regulators of cell division and extension. Our previous findings have highlighted the positive effects of MWCNTs on plant growth and development.[12,13] The current data highlight the novel positive effects of MWCNTs at the cellular level and provide an understanding of the complex mechanism underlying the enhancement of plant growth. Applications may include enhanced production of plant cell cultures (suspensions, callus cultures) for the pharmaceutical industry, agro-biotechnology, or the bioenergy industry. However, to consider the possible use of carbon nanoparticles in the food sector of agriculture, the consequences of the introduction of carbon nanotubes into the environment have to be thoroughly investigated. The detailed assessment of the potential environmental risks of using carbon nanomaterials in agriculture is a requirement in order to fully understand the positive or negative impact that such engineered nanomaterials may have on agriculture. The data presented in this manuscript could further stimulate research focused on understanding the effects of carbon nanotubes on cell proliferation and on monitoring the expression of genes and proteins involved in cell division and water transport in other cell systems, such as those found in bacteria, animals, and fungi. The discovery of the mechanisms behind the effects that nanomaterials have on the transcriptome and proteome of different types of cells is important for basic science and could be beneficial in the development of new biotechnologies.

METHODS

Establishment of Callus Tobacco Culture. Seeds of tobacco (*cv. Havana*) were sterilized and germinated on Murashige and Skoog medium (MS) with 0.8% agar. Leaves of 3-week-old sterile tobacco seedlings were cut and placed on MS medium supplemented with 2% sucrose and 1 mg/L of 2,4-D. For callus induction, leaf explants were cultivated in dark conditions for 1 month. The established calli were excised from leaves and transferred to fresh MS medium (0.8% agar, 2% sucrose, 1 mg/L 2,4-D). The callus cultures were maintained at 22−24 °C in the dark inside a tissue culture growth chamber.

Experiments with Established Tobacco Cell Culture and Statistical Analysis. Special tubes for plant cell culture (Phytotechnology Laboratories, Inc.) were used for experiments with established tobacco callus. The basic medium for callus cultivation was MS medium supplemented with 1 mg/L 2,4-D. This medium was used as control. For experimental conditions, the control medium was supplemented with MWCNTs in concentrations of 0.1, 5, 100, and 500 μg/mL or activated carbon in the same concentrations: 0.1, 5, 100, and 500 μg/mL. An equal amount of initial callus (inoculum), 300 mg, was placed in each experimental tube containing agar medium with or without carbonaceous material. Experimental tubes were kept in dark conditions at 22−24 °C in a growth chamber for 1 month. Each experimental condition was replicated 10 times (10 tubes were used for each concentration of carbon nanotubes, activated carbon, or control medium). All of the experiments were repeated twice; therefore, each data point is the average of 20 individual measurements. Thus, vertical bars indicate ± SE (n = 20).

Synthesis and Processing of MWCNTs. High yield and crystalline multiwalled carbon nanotubes (MWCNTs) were synthesized by chemical vapor deposition connected to a radio-frequency (RF) generator with a 350 kHz frequency as previously reported.[34−37] Produced MWCNTs were purified with a diluted hydrochloric acid under continuous agitation.[34] Subsequently, the sample was washed with DI water to ensure complete removal of the acid. The cleansed nanotubes were found to have a purity of 98%, while still remaining free of defects. TEM analysis indicated that the MWCNTs used in this work had an average diameter of 20 nm and lengths ranging from 500 nm to 1 μm.

Raman Spectroscopy. The tobacco cells grown for 1 month on MS medium supplemented with 100 μg/mL of MWCNTs were used for Raman-scattering analysis. The cells that did not have direct contact with the MWCNTs-containing medium (top of callus) were carefully collected and analyzed. Raman-scattering analysis was performed at room temperature with a Horiba Jobin Yvon LabRam HR800 spectrometer equipped with a charge-coupled detector and two grating systems (600 and

1800 lines/mm). A 633 nm (1.96 eV) laser excitation was used for these studies. The laser beam intensity at the sample surface was 20 mW and was focused through an Olympus microscope to a spot size of <1 μm^2. The backscattered light was collected in a 180° geometry from the direction of incidence. Raman shifts were calibrated on a silicon wafer at the 521 cm^{-1} peak.

Transmission Electron Microscopy (TEM) of Tobacco Cells. Callus samples (upper part of calluses grown on regular MS medium and calluses grown on medium supplemented with MWCNTs (100 μg/mL)) were carefully removed from the surface of the agar with a spatula, placed on dental wax, cut into 1 mm cube blocks with a razor blade, and fixed in 3% glutaraldehyde in 0.075 M Sorensen's buffer, pH 7.2 for 42 h at 4 °C. Thereafter, callus pieces were washed three times for 30 min each in 0.075 M Sorensen's buffer, pH 7.2 at 4 °C. Samples were then postfixed in 1% osmium tetroxide in 0.075 M Sorensen's buffer, pH 7.2 for 2 h at 4 °C in the dark. Callus pieces were again washed three times for 30 min each in 0.075 M Sorensen's buffer, pH 7.2 at 4 °C, followed by dehydration in a graded ethanol series (30%, 50%, 70%, 95%, and 3 × 100%) in 30-min to 1-h increments beginning at 4 °C and warming to room temperature in 100% ethanol. Callus pieces were infiltrated with Spurr's resin over several days and cured at 70 °C overnight. Thin sections were cut from the embedded samples using an ultramicrotome equipped with a diamond knife and were mounted on copper grids. The grids were stained with uranyl acetate and lead citrate before examination with a transmission electron microscope (JEOL 1200EX). Images were captured with Kodak 4489 film which was subsequently scanned using an Epson Perfection 4870 Photo flatbed scanner at 1200dpi. Scanned images were processed and labeled using Photoshop CS4.

Real-Time PCR Analysis. Total RNA samples from tobacco cells incubated on regular MS medium, MS medium supplemented with MWCNTs (100 ug/mL), or with activated carbon (100 ug/mL) at the initial stage of incubation (0 days) and at different time points of incubation (6 h, 24 h, 4 days, 8 days, 16 days, 25 days) were isolated using RNeasy Plant Mini Kit (Qiagen Inc. Valencia, CA). Residual DNA was removed by on-column DNA digestion using the RNase-free DNase Kit (Qiagen Inc. Valencia, CA). Synthesis of cDNA was carried out using SuperScript III First Strand Synthesis System Kit (Invitrogen, Carlsbad, CA) with dT16-oligonucleotide primers according to the manufacturer's protocol. *NtLRX1* (extensin1) gene (AB273719) was amplified using 5′-AGCCACCACCATACACACCTCAAT-3′ (forward primer) and 5′-TGGTGGTGAAGACGGTGTCACATA-3′ (reverse primer); *NtPIP1* (AF440271) gene was amplified using 5′-GGTTCATTTGGCCAC-CATCCCAAT-3′ (forward primer) and 5′-GCAGCAAGAGCAGCTC-CAATGAAT-3′ (reverse primer); *CycB* gene (AY776171) was amplified using 5′-TTCTGGCTGAGCTGGGATTGATGA-3′ (forward primer) and 5′-TGATGGTGTGTCGAGCAGCATAGA-3′ (reverse primer); *actin* gene (AB158612) as the internal control was amplified using 5′-GAACGGGAAATTGTCCGCGATGTT-3′ (forward primer) and 5′-ATGGTAATGACCTGCCCATCTGGT-3′ (reverse primer). The quantification of expression of all tested genes during 25 days of cell incubation was conducted by real-time quantitative RT-PCR analysis (qRT-PCR) using SYBR Green PCR master mix (Applied Biosystems, Carlsbad, CA, USA) in an iCycler iQ Multi Color Real Time PCR detection system (Bio-Rad, Hercules, CA, USA). Three independent biological replicates were used in the analysis. The real-time PCR data were generated and analyzed by the "comparative count" method to obtain the relative mRNA expression of each tissue as described in the iCycler manual (Bio-Rad). Actin was chosen as an internal control based on the equal amplification efficiencies of actin and all analyzed genes (*CycB, NtLRX1, NtPIP1*). Data for the amplification efficiencies of actin and gene of aquaporin (*NtPIP1*) are shown in the Supporting Information, Figure S3.

Immunoblot Analysis. For analysis of the production of tobacco water channel (aquaporin) protein PIP1, the total protein was extracted from cells incubated on regular MS medium, MS medium supplemented with MWCNTs (100 ug/mL), or supplemented with activated carbon (100 ug/mL) at the initial stage of incubation (0 days) and at different time points of incubation (6 h, 24 h, 4 days, 8 days, 16 days, 25 days) using the Plant Total Protein Extraction Kit (Sigma-Aldrich, Inc., St. Louis, MO). The analysis of the production of aquaporin protein in tobacco cells was performed using antipeptide antibodies provided by Pacific Immunology, Inc. (Ramona, CA). Antibodies were designed and produced against the tobacco aquaporin peptide sequence DAKRNARDSHV. Standard techniques for Western blot analysis[38,39] were used for the detection of tobacco water channel protein (NtPIP1) in tobacco cells exposed and unexposed to carbonaceous materials. Briefly, for each time point, 20 μg of total protein was separated by SDS-PAGE followed by Western analysis using affinity purified anti-PIP1 primary and antirabbit IgG HRP secondary antibodies. Equal loading of the protein was assessed using antiactin primary and antimouse IgG HRP secondary antibodies.

Conflict of Interest: The authors declare no competing financial interest.

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Supporting Information Available: Additional data related to monitoring the dry weight of callus biomass, real-time PCR assay, assessment of tobacco aquaporin gene and actin for equal amplification efficiencies. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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