Plant Physiology and Biochemistry 129 (2018) 122–129

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

The effect of graphene oxide on adventitious root formation and growth in apple

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ARTICLE INFO

Keywords:
Adventitious root
Gala
Gene expression
Graphene oxide
Growth

ABSTRACT

Graphene, a new type of nanomaterial, has unique physical properties and important potential biological applications. However, few studies have been conducted on the environmental impact of graphene. Therefore, to explore the effect of graphene on plants, three-week-old, tissue-cultured 'Gala' apple plants (*Malus domestica*) were treated with different concentrations (0, 0.1, 1, 10 mg/L) of graphene oxide (GO) and examined after 40 days. Results indicated that adventitious root length, moisture content and the number of lateral roots were all inhibited by 0.1–10 mg/L GO. At 0.1 and 1 mg/L GO, however, the number of adventitious roots and the rooting rate exhibited a significant increase, relative to the control (no GO). Treatment with GO increased the activities of oxidative stress enzymes including catalase (CAT), peroxidase (POD), and superoxide dismutase (SOD) in the apple plants, relative to controls. Malondialdehyde (MDA) levels were also significantly decreased at 10 mg/L GO. Treatment of apple plantlets with 0.1 mg/L GO increased the transcript abundance of auxin efflux carrier (*PIN7, ABCB1*) genes and auxin influx carrier (*LAX2, LAX3*) genes but inhibited the transcript levels of the *ARR3* gene, which involved in cytokinin biosynthesis. Additionally, the transcript levels of *ARRO1*, *ARF19*, and *TTG1*, which play roles in the formation of adventitious roots, lateral roots, and root hairs, respectively, were all decreased in response to treatment with 1 and 10 mg/L GO. Collectively, the results indicate that treatment of 'Gala' apple plants with 0.1 mg/L GO had a positive effect on root formation but a negative effect on root growth. This response may be related to the negative impact of GO on cellular structure and function.

1. Introduction

Nanomaterials can be defined as materials in which a single unit is between 1 and 1000 nm (10^{-9} m) in size (in at least one dimension) but usually 1–100 nm (Buzea et al., 2007). Compared to traditional structural materials, nanomaterials have unique properties characterized by high hardness, high toughness, and super plasticity. Nanomaterials are now widely used in energy, medicine, cell and tissue culture engineering, electronics, development of new materials, and environmental protection (Lin et al., 2014; Lü et al., 2013; Mura and Couvreur, 2012; Parvez et al., 2013; Sharma, 2014; Zhang et al., 2012).

Graphene and its derivatives have received considerable interest since the discovery of graphene in 2004 (Geim and Novoselov, 2007). Researchers have made considerable progress in understanding their physical, chemical, and biological properties (Service, 2009). Graphene oxide (GO), one of the derivatives of graphene, has a unique two-dimensional layered structure which is only one atom thick. GO is composed of oxygenated functional groups and has a high specific surface area, comprehensive surface accessibility, and marginal reactivity (Zubir et al., 2014).

There are few studies on the potential application of nanomaterials in agriculture. Exploring the effect of nanomaterials on plant growth and development and its mechanism of action is necessary to evaluate their potential application in agriculture. Studies have shown that nanomaterials can affect seed germination, root development, and the development of aboveground plant parts (Begum et al., 2011; Ratnikova et al., 2015). The initial mechanism of action that has been proposed is that the nanomaterials enter plant tissues and cells and affect the antioxidant enzyme system in plants (Moller et al., 2007) and cellular metabolism (Hu et al., 2014). Nanomaterials (carbon nanotubes, C60, graphene) have been reported to affect seed germination and root growth in tomato (Khodakovskaya et al., 2009) and corn (Lin and Xing, 2007), increase the rate of seed germination in rice, and the overall vitality and health of rice seedlings (Nair et al., 2012). No studies have been conducted, however, on the effect of nanomaterials on the growth and development of apples.

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https://doi.org/10.1016/j.plaphy.2018.05.029
Received 7 March 2018; Received in revised form 29 May 2018; Accepted 29 May 2018
Available online 30 May 2018

Apple (*Malus domestica*) is one of the most widely cultivated fruit crops in the world and is an important part of a healthy human diet. Abiotic (low temperature, high salinity, heavy metals) and biotic stresses (diseases and insect pests) can cause huge losses in apple productivity and yield in China and other apple growing regions. Therefore, it is important to explore the effects of nanomaterials on apple growth and development to determine if they can be used as a management tool to deal with apple production problems.

The objective of the present study was to determine the effect of graphene oxide on root formation and development in apple. 'Gala' apple has good regenerative properties and so has been, widely used in apple transformation research (Dai et al., 2013); therefore, 'Gala' was selected for use in the current study. Results indicated that treatment of apple plantlets with different concentration of GO significantly affected root formation and development.

2. Materials and methods

2.1. Graphene oxide (GO)

A liquid preparation of GO particles was obtained from the Nanjing XFNANO Materials Tech Co., Ltd., China with the following characteristics: particle diameter of 50–200 nm, a single layer ratio > 99%, a thickness ranging from 0.8 to 1.2 nm, and a purity of 99%. The GO particles were immersed in water, and the material was stored in a tightly sealed container in the dark at temperatures less than 20 °C.

2.2. Plant materials

Tissue-cultured plants of 'Gala' apple (*Malus domestica*) were subcultured in Murashige and Skoog (MS) medium (Toshio Murashige and Skoog, 1962) containing 0.3 mg/L 6-benzyladenine (BA), 0.2 mg/L indoleacetic acid (IAA) and 0.1 mg/L GA3, at 25 °C under a 16/8-h (day/night) photoperiod and subcultured every 4 weeks.

2.3. Effects of GO on the growth and development of apple plants

Three-week-old 'Gala' plants with uniform growth, were used for the experiment. The plants were transferred into 1/2 MS medium supplemented with 1 mg/L IAA, 0.4 mg/L indolebutyric acid (IBA), and different concentrations of GO in glass jars. GO was added to autoclaved medium at concentrations of 0.1, 1, and 10 mg/L after the GO solution had been sonicated for 30 min. Culture medium without GO was used to grow control plants. The plants were placed in a growth chamber at 25 ± 1 °C, with a photoperiod of 16 h of light and 8 h of dark. At least three plants were randomly selected at each of the tested GO concentrations and examined after 40 days of being transferred to the GO medium. The effect of the different GO concentrations on adventitious root formation was also examined with the aid of stereomicroscope (Leica S8APO) at earlier time points (10th, 12th, 19th day). The adventitious roots were cut at their base and weighed to determine adventitious root fresh weight.

Adventitious root length was defined as the length from the root tip to the base of the root. The number of adventitious roots and laterals was also recorded. Root fresh weight and dry weight (after drying at 65 °C for 48 h) were also determined. Moisture content and rooting rates were calculated as (fresh weight – dry weight)/fresh weight and (number of rooting plants in a treatment/number of total plants in a treatment), respectively. The number of adventitious roots per plant and the number of lateral roots on each adventitious root were recorded. The diameter of adventitious roots was measured at the base of the root with a vernier caliper.

Table 1
Primers for qRT-PCR.

Gene name	Primers	Sequence	Annealing temperature (°C)
Tublin	TublinF	5′-AGGATGCTACAGCCGATGAG-3′	56
	TublinR	5-GCCGAAGAACTGACGAGAATC-3	
PIN7	PIN7F	5′-TTTGGGTTTTATCCCGCGCAGA-3′	60
	PIN7R	5′-CGGCGGCTGCCTGATTTTTGC-3′	
ABCB1	ABCB1F	5′-ACTCTCGTCATTGCCCATCG-3′	60
	ABCB1R	5′-AGCTTGGCATAGACACCGTT-3′	
LAX2	LAX2F	5′-CTACCACCACCACATCAC-3′	60
	LAX2R	5′-GGAAACACCACCAATTCAC-3′	
LAX3	LAX3F	5′-GTGGTTTGAAGTGTTGGATGGT-3′	60
	LAX3R	5′-GGGGATGAAGACTGTGGTGG-3′	
ARR3	ARR3F	5′-TGATCCGACGATTGCTCCATC-3′	60
	ARR3R	5′-CAGCGTTGGTCATTTTCAACC-3′	
ARRO1	ARRO1F	5′-GAACCACATGATCCCACCGGCT-3′	60
	ARRO1R	5′-ATCCACTGCCCGCTATCACATCCT-3′	
ARF19	ARF19F	5′-CTCCTACCAGAAGCAGATCAAA-3′	60
	ARF19R	5′-GCAGTAAGTGTCTTGCAGAATG-3′	
TTG1	TTG1F	5′-ACAGCAAGACCAGCGAGTTT-3′	60
	TTG1R	5′-CAGCCGAAACCGAAGCAAAA-3′	

2.4. Reverse-transcription quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from sampled roots using a Plant Total RNA Isolation Kit (Foregene) according to the manufacturer's instructions. First-strand cDNA was synthesized using the PrimeScript RT reagent Kit with gDNA Eraser (TAKARA). RT-qPCR was carried out on an ABI 7300 Real-Time PCR System (Applied Biosystems), using the SYBR Premix Ex Taq reaction system (TAKARA). Each analysis consisted of three biological replicates and three technical replicates of each biorep. The apple *Tubulin* gene was used as the internal control to normalize sample variance. Final results were expressed as the relative expression ratio between the targeted gene and the reference gene. The designed gene-specific primers used in the RT-qPCR analysis are listed in Table 1.

2.5. MDA Content and antioxidant enzyme activity

To measure malondialdehyde (MDA) and enzyme activity, 0.2 g of frozen root material was homogenized in 2 mL of 50 mM phosphate buffer (PBS) (pH 7.8, 4 °C) containing 0.2 mM ethylene diamine tetraacetic acid (EDTA) and 1% (w/v) polyvinyl pyrrolidone (PVP). The homogenate was then centrifuged at 12,000 × g for 20 min at 4 °C. Subsequently, 0.5 mL of supernatant was added to 2 mL of 10% (w/v) trichloroacetic acid (TCA) and 2 mL of 0.5% (w/v) 2-thiobarbituric acid (TBA). The mixture was heated in a water bath shaker at 95 °C for 30 min. The samples were then rapidly cooled in an ice-bath and centrifuged at 8000 rpm for 10 min. Absorbance at 450, 532, and 600 nm was then recorded. The concentration of MDA was expressed as μmol/g fresh weight (FW). Three replicates were utilized to make each of the measurements.

To determine total superoxide dismutase (SOD) activity, 3 mL of reaction solution containing 50 mM PBS (pH 7.8), 13 μM methionine, 63 μM nitroblue tetrazolium (NBT), 1.3 μM riboflavin, and 30 μL of supernatant were incubated for 30 min under fluorescent light at 25 °C. The absorbance of the solution was subsequently measured at 560 nm. One unit of SOD was the amount of extract that reduced the rate of NBT reduction by 50% (Pinhero et al., 1997). Three replicates were utilized to make each of the measurements.

One unit of catalase (CAT) activity was defined as the amount of H_2O_2 (μmol) depleted per min at 240 nm. Changes in absorbance were read every 10 s for 6 min in a 3 mL reaction solution containing 1.95 mL distilled water, 1 mL 0.3% H_2O_2(v/v), and 50 μL of supernatant. Three replicates were utilized to make each of the measurements.