

Titanium dioxide nanoparticles affect the growth and microRNA expression of tobacco (*Nicotiana tabacum*)

Taylor P. Frazier · Caitlin E. Burklew · Baohong Zhang

Received: 28 June 2013 / Revised: 30 August 2013 / Accepted: 30 September 2013 / Published online: 15 October 2013
© Springer-Verlag Berlin Heidelberg 2013

Abstract Titanium dioxide (TiO₂) is one of the most widely used pigments in the world. Due to its heavy use in industry and daily life, such as food additives, cosmetics, pharmaceuticals, and paints, many residues are released into the environment and currently TiO₂ nanoparticles are considered an emerging environmental contaminant. Although several studies have shown the effect of TiO₂ nanoparticles on a wide range of organisms including bacteria, algae, plankton, fish, mice, and rats, little research has been performed on land plants. In this study, we investigated the effect of TiO₂ nanoparticles on the growth, development, and gene expression of tobacco, an important economic and agricultural crop in the southeastern USA as well as around the world. We found that TiO₂ nanoparticles significantly inhibited the germination rates, root lengths, and biomasses of tobacco seedlings after 3 weeks of exposure to 0.1, 1, 2.5, and 5 % TiO₂ nanoparticles and that overall growth and development of the tobacco seedlings significantly decreased as TiO₂ nanoparticle concentrations increased. Overall, tobacco roots were the most sensitive to TiO₂ nanoparticle exposure. Nano-TiO₂ also significantly influenced the expression profiles of microRNAs (miRNAs), a recently discovered class of small endogenous noncoding RNAs (~20–22 nt) that are considered important gene regulators and have been shown to play an important role in plant development as well as plant tolerance to abiotic stresses such as drought, salinity, cold, and heavy metal. Low concentrations (0.1 and 1 %) of TiO₂ nanoparticles dramatically induced miRNA expression in tobacco seedlings with miR395 and miR399 exhibiting the greatest fold changes of 285-fold and 143-fold, respectively. The results of this study show that TiO₂ nanoparticles have a negative impact

on tobacco growth and development and that miRNAs may play an important role in tobacco response to heavy metals/nanoparticles by regulating gene expression.

Keywords Titanium dioxide · Nanoparticle · Phytotoxicity · MicroRNA · Gene expression · *Nicotiana tabacum* · Tobacco

Introduction

Titanium dioxide (TiO₂) is a common whitening agent used in the production of foods, sunscreens, cosmetics, plastics, medicines, papers, pharmaceuticals, and paints. Estimates have shown that approximately 4 million metric tons of bulk TiO₂ is produced annually around the globe with the USA leading production with 1.3 million metric tons (Lubick 2009). TiO₂ production also accounts for 70 % of the total amount of manufactured pigments worldwide (Baan et al. 2006; Trouiller et al. 2009). TiO₂ is becoming increasingly used in the industry due to its white pigment, ability to block ultraviolet light, and photocatalytic properties that are commonly used to destroy pollutants in soil, air, and water/wastewater treatment (Aarthi and Madras 2007; Higarashi and Jardim 2002).

Due to its heavy use in the industry and daily life, many TiO₂ residues are released into the environment, and currently, TiO₂ nanoparticles are being considered an emerging environmental contaminant. Studies analyzing the health effects of TiO₂ nanoparticle exposure have led it to be classified by the International Agency for Research on Cancer as a group 2B carcinogen, meaning that it is potentially harmful to humans (Baan et al. 2006). Accordingly, mounting evidence has shown that TiO₂ nanoparticles create lipid peroxidation products and reactive oxygen species that induce epithelial cell injury and oxidative DNA damage in cell lines, mice, and rats (Falck et al. 2009; Trouiller et al. 2009). Since TiO₂ nanoparticles are a

T. P. Frazier · C. E. Burklew · B. Zhang (✉)
Department of Biology, East Carolina University, Greenville,
NC 27858, USA
e-mail: zhangb@ecu.edu

potential hazard to human health, studies in rat models have shown that lung cancer can be induced after exposure to nano-TiO₂ (Pott and Roller 2005). It should be noted, however, that no studies have shown a correlation between TiO₂ nanoparticle exposure and the development of lung cancer in industry workers (Boffetta et al. 2004; Fryzek et al. 2003).

As the manufacturing and industrial use of TiO₂ increases, the amount of TiO₂ nanoparticles released into the environment is also likely to increase. This is due in part to the high use of TiO₂ and the ability of TiO₂ nanoparticles to travel freely through wastewater treatment plants and enter neighboring soils and streams (Kiser et al. 2009; Lubick 2009). Therefore, TiO₂ is also being investigated as a potential environmental contaminant and the effect of TiO₂ in water (Adams et al. 2006; Kaegi et al. 2008) and on soils (Higarashi and Jardim 2002; Mattigod et al. 2005), bacteria (Kubo et al. 2005; Wang et al. 2009), algae (Hartmann et al. 2009; Hund-Rinke and Simon 2006), fungi (Sichel et al. 2007), plankton (Hund-Rinke and Simon 2006; Lovern and Klaper 2006; Lovern et al. 2007), and fish (Federici et al. 2007; Reeves et al. 2008) have been analyzed. A recent study has also observed that TiO₂ nanoparticles cause genetic toxicity to *Salmonella typhimurium* (Pan et al. 2010). It is surprising, given the increasing concern of TiO₂ nanoparticles to the environment, that only two studies have analyzed the effect of TiO₂ nanoparticles on plants (Asli and Neumann 2009; Zheng et al. 2005), and no study has been performed on understanding the effect of nanoparticles on regulatory RNAs.

microRNAs (miRNAs) are a recently discovered class of small regulatory endogenous RNAs (~20–22 nt) that do not code for proteins (Bartel 2004; Zhang et al. 2006). miRNAs play an important role in plant growth and development as they have been shown to aid in leaf development (Juarez et al. 2004), stem and root growth (Laufs et al. 2004; Mallory et al. 2004), organ maturation (Achard et al. 2004; Aukerman and Sakai 2003; Chen et al. 2004; Mlotshwa et al. 2006), and the plant's ability to withstand abiotic stresses (Jones-Rhoades and Bartel 2004; Sunkar and Zhu 2004). miRNAs target messenger RNAs (mRNAs) and regulate gene expression by marking these mRNAs for degradation or repressing protein translation (Bartel 2004; Vaucheret 2006). Recent studies have shown that miRNAs mediate the expression of more than 30 % of genes coding for protein (Lewis et al. 2005; Xie et al. 2005), and this number is expected to rise as more miRNAs and their target sequences are identified.

miRNAs have been shown to play an important role in many biological and metabolic processes. Specifically, certain miRNAs have been identified in plant responses to abiotic stresses. For example, miR156, miR169, miR395, miR399, and miR399* (miR399* is the opposite strand of miR399 in the pre-miRNA stem-looped structure) expression levels are altered during the response of *Arabidopsis* to phosphate starvation (Fujii et al. 2005; Hsieh et al. 2009). miR398 and miR408

have been shown to be upregulated in drought-exposed *Medicago truncatula* plants (Trindade et al. 2010), and miR398 has been implicated in plant response to copper and oxidative stress (Abdel-Ghany and Pilon 2008; Sunkar et al. 2006). miRNA expression has also been shown to be altered under both cold (Zhang et al. 2009; Zhou et al. 2008a) and salt (Ding et al. 2009; Jia et al. 2009) stresses.

In this study, we used tobacco as a model species to analyze the effect of TiO₂ nanoparticles on the growth, development, and miRNA expression levels of an agricultural plant. Tobacco is an important economic and agricultural crop in the southern USA as well as around the world. Although much research has been dedicated to this crop, no experiments have shown the effects of TiO₂ nanoparticles on tobacco growth and development. We found that as the concentration of nano-TiO₂ increased, the germination rates, root lengths, biomasses, and leaf counts of 3-week-old tobacco seedlings decreased in a concentration dependent manner. We also found that the expression levels of miRNAs were either up- or downregulated in response to increasing TiO₂ nanoparticle concentrations. Ultimately, we were able to conclude that TiO₂ nanoparticles negatively affect tobacco growth and that miRNAs may play an important role in tobacco plants response to heavy metal toxicity, including nanoparticles.

Materials and methods

Tobacco treatment

Tobacco (*Nicotiana tabacum*) seeds were sterilized in 70 % ethanol for 2 min followed by 10 % bleach for 15 min. The seeds were then rinsed with sterile distilled water until no bleach odor remained. Sterilized seeds were sowed on Petri dishes containing Murashige and Skoog (MS) medium containing Gamborg's B5 vitamins (0.44 % salts, 1 % sucrose, 0.8 % agar, pH 5.8) and 0, 0.1, 1, 2.5, or 5 % TiO₂ nanoparticles. TiO₂ nanoparticles were purchased from Sigma. The particle size is less than 25 nm with more than 99.5 % purity. A total number of 25 seeds were sowed per plate, and there were a total number of 5 plates for the control and each treatment with different TiO₂ nanoparticle concentrations. The plates were inverted at a 75° angle to promote downward root growth and allowed to grow for 3 weeks on a 16-h day/8 h night cycle.

Measuring germination rate, leaf count, root length, and biomass

After 3 weeks of growth, the germination rate of all five plates for the control and each concentration of nano-TiO₂ was recorded. We followed the procedure as described in Lin and Xing (2007), in which we recorded a seed germinated if the

radical or cotyledon was observed protruding from the seed coat. The number of leaves per seedling per plate for the control and different concentrations of TiO₂ was also recorded. Seedlings were removed from the media, and the root lengths of the control, 0.1 % TiO₂, and 1 % TiO₂ plates were measured (in centimeters) using a standard ruler. The root lengths of 2.5 and 5 % seedlings were not measured because no roots were formed. In the case where more than one root was formed, all roots were measured, but only the largest root measurement was used in calculating average root length. Once root lengths were recorded, seedlings were then placed on a weigh boat and the weight of biomass measured using a Discovery 214C balance (Ohaus, Pine Brook, NJ). After the biomass of each plate was recorded, the seedling tissue was immediately placed in liquid nitrogen and stored at -80 °C until RNA extraction.

Total RNA extraction

Total RNA was isolated from the 3-week-old seedlings using the mirVana miRNA Isolation Kit (Ambion, Austin, TX) according to the manufacturer's protocol. Total RNA was then quantified and assessed for quality using a Nanodrop ND-1000 (Nanodrop Technologies, Wilmington, DE). RNA samples were stored at -80 °C until further analysis.

Analyzing microRNA expression changes using RT-PCR and qRT-PCR

Quantitative real-time PCR (qRT-PCR) has become one of the most mature techniques to detect the miRNA expression in different organisms; this is because it can be used to distinguish one single nucleotide change during the miRNA sequence, and it is highly sensitive to miRNA expression (Chen et al. 2005). This technique has been used to measure and validate miRNA expression in many plant species. Thus, in this study, Applied Biosystems TaqMan microRNA Assays were employed to detect and quantify tobacco miRNAs using stem loop real-time PCR according to the manufacturer's instructions. There were two steps in the TaqMan miRNA Assays: (a) reverse transcription of the mature miRNA to a longer single-stranded cDNA sequence using a miRNA-specific stem-looped primer and (b) quantitative real-time PCR. Briefly, a single-stranded miRNA cDNA was generated from 1 µg of the total RNA from the control, 0.1 %, and 1 % TiO₂ nanoparticle tissue samples by reverse transcription using the Applied Biosystems TaqMan microRNA Reverse Transcription Kit and miRNA-specific stem-looped RT primers. Each reaction was repeated in triplicate. In total, we investigated changes in the expression levels of 11 different miRNAs (miR156, miR159, miR162, miR167, miR169, miR172, miR393, miR395, miR396, miR398, and miR399) and 2 stress-related genes (alcohol dehydrogenase (ADH)

and alcohol peroxidase (APX)). The reason for selecting these 11 miRNAs is because these 11 miRNAs were aberrantly expressed during abiotic stresses in model plant species or play important role in plant growth and development (Khraiwesh et al. 2012; Zhang et al. 2006). Two housekeeping genes (elongation factor 1 (EF1) and tubulin) were used as reference genes to normalize expression values. All reactions were performed in triplicate, and the results were analyzed using the $\Delta\Delta C_T$ method.

Results

Nano-TiO₂ inhibited tobacco seed germination and plant growth

After 3 days of planting, seeds started to germinate. At low concentrations (0.1 and 1.0 %), nano-TiO₂ did not affect tobacco seed germination (Fig. 1a). However, nano-TiO₂ significantly affected tobacco seed germination at higher concentrations ($p=0.016$). When 2.5 % TiO₂ nanoparticles were added into the MS medium, the germination rate of seeds decreased from 98–100 to 93.60 ± 3.58 %. Also, the germination of tobacco seeds was completely inhibited by exposure to 5.0 % nano-TiO₂. Although tobacco seeds germinated with a higher percentage after exposure to 2.5 % nano-TiO₂, it was hard for these seeds to produce roots and fully developed cotyledons. Therefore, the germinated seedlings quickly died within about 1 week.

After 3 weeks, there was a significant obvious decrease in plant growth and development after exposure to elevated TiO₂ nanoparticle concentrations as evidenced by decreases in root lengths ($p<0.001$), leaf numbers ($p<0.001$) as well as plant biomasses ($p<0.001$) (Figs. 1 and 2). Roots were the most sensitive to TiO₂ nanoparticle exposure (Fig. 1b). Under normal conditions, the root lengths reached to 1.43 ± 0.15 cm after 3 weeks of culture; however, after exposure to 0.1 and 1 % nano-TiO₂, the root lengths were significantly reduced to 0.74 ± 0.05 and 0.21 ± 0.04 cm ($p<0.001$), respectively. A 2.5 % nano-TiO₂ completely inhibited the appearance and elongation of roots. An interesting phenomenon is that TiO₂ nanoparticle exposure influenced differential root patterns. Tobacco is a dicot; generally speaking, one seed only produces one major root. However, after exposure to 1.0 % TiO₂, a majority of tobacco seedlings produced two roots, although both were very short with an average of 0.21 ± 0.04 cm after 3 weeks of growth. One of the potential reasons is that nano-TiO₂ exposure can damage the major root apical meristem and therefore allow the seedlings to produce an additional root from the damaged location (Fig. 2c).

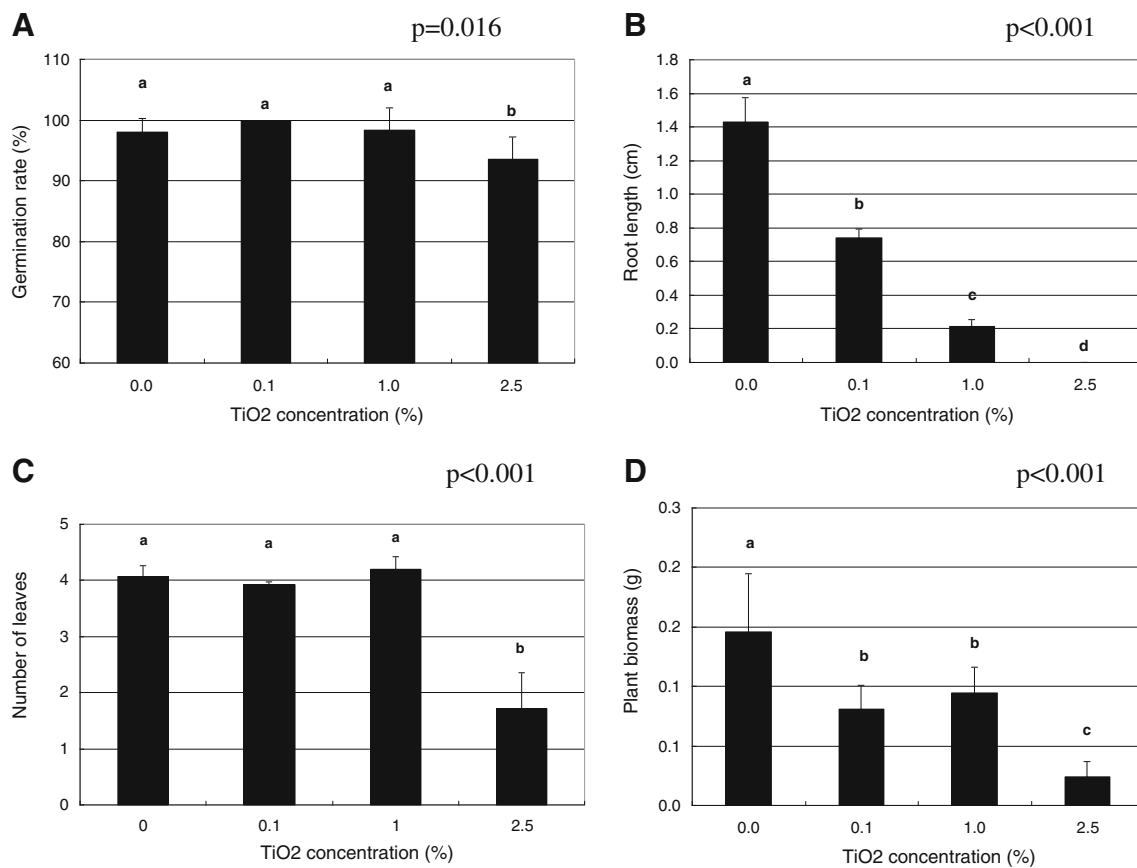


Fig. 1 Effect of nanoparticle TiO₂ on tobacco seed germination, growth, and development. **a** Germination rate, **b** root length, **c** total number of leaves, and **d** plant biomass

Exposure to nano-TiO₂ also influenced tobacco leaf development as demonstrated by a reduction in the total number of leaves and the leaf size. After exposure to nano-TiO₂, tobacco leaves grew much smaller compared to the untreated control (Fig. 2). The averages for total leaf number were 4.07 ± 0.19 , 3.92 ± 0.06 , 4.19 ± 0.24 , and 1.72 ± 0.63 for the control, 0.1 % nano-TiO₂, 1 % nano-TiO₂, and 2.5 % nano-TiO₂ groups, respectively (Fig. 1c). Although TiO₂ nanoparticle exposure significantly reduced the total number of seedling leaves at higher concentrations (2.5 %; $p < 0.001$), no significant differences were

observed between the untreated control and low nano-TiO₂ treatments ($p = 0.536$ for 0.1 % and $p = 0.626$ for 1 %).

TiO₂ nanoparticle exposure significantly reduced plant growth and biomass even at very low concentrations (Figs. 1d and 2; $p < 0.001$). For the untreated control, the average biomass was 146 ± 49 mg. Nano-TiO₂ significantly inhibited tobacco plant growth, and plant biomass was reduced to 81 ± 21 mg after exposure to 0.1 % nano-TiO₂ ($p < 0.001$). However, there was no significant difference in plant biomass between the 0.1 and 1 % TiO₂ nanoparticle

Fig. 2 Effect of nano-TiO₂ on tobacco growth and development after 3 weeks of treatment. **A** MS media (control). **B** MS+0.1 % nano-TiO₂. **C** MS+1 % nano-TiO₂. **D** MS+2.5 % nano-TiO₂. Figure 2c shows that multiple roots were generated from the damaged sites



treatments ($p=0.431$). When 2.5 % nano-TiO₂ was added into the medium, almost all seedlings died within 1 week of germination and minimal plant biomass was harvested (Fig. 1d).

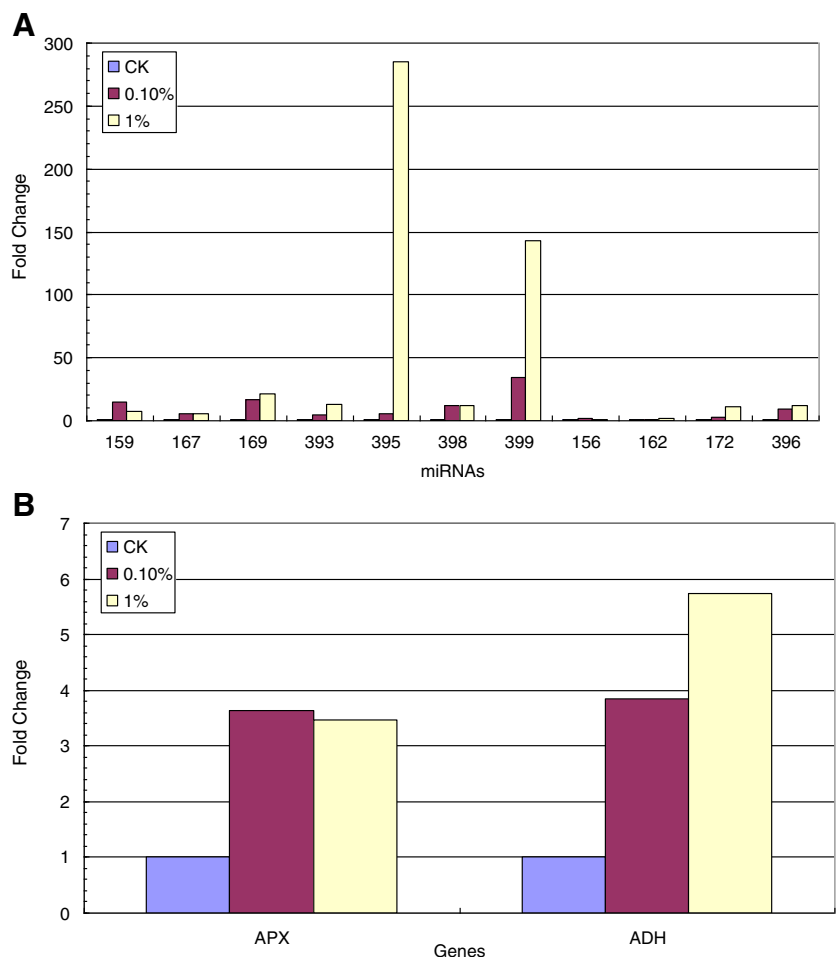
TiO₂ nanoparticles affect miRNA expression in tobacco

TiO₂ nanoparticle exposure significantly affected miRNA expression (Fig. 3). Among the 11 miRNAs investigated in this study, the miRNA expression levels were upregulated at both concentrations (0.1 and 1.0 %) after nano-TiO₂ exposure for all miRNAs except miR156 at high concentration (1.0 %). According to their response to nano-TiO₂ exposure, these 11 miRNAs could be classified into three groups: (1) miRNAs whose expression levels were increased as TiO₂ nanoparticle concentrations increased, the majority of tested miRNAs belonged to this group, which included miR169, miR393, miR395, miR399, miR172, and miR396; (2) miRNAs whose expression levels decreased as TiO₂ nanoparticle concentrations increased, there are two miRNAs (miR159 and miR156) that belonged to this group; and (3) miRNAs whose expression levels were not significantly changed as TiO₂ nanoparticle

concentrations increased, there are three miRNAs (miR162, miR167, and miR398) that belonged to this group.

Among the 11 tested miRNAs, miR395 and miR399 were the two most sensitive to nano-TiO₂ exposure, exhibiting the greatest fold changes to 285-fold and 143-fold, respectively. This suggests that these two miRNAs play an important function in tobacco seedlings facing nanoparticle or metal stress. It was also observed that these two miRNAs were upregulated under different environmental abiotic stresses, such as drought and salinity stress in other plant species (Jones-Rhoades and Bartel 2004; Zhao et al. 2007). Six miRNAs (miR169, miR159, miR393, miR398, miR396, and miR172) are moderately sensitive to nano-TiO₂ exposure, exhibiting at least a 10-fold change after 3 weeks of growth on the medium containing 0.1 or 1.0 % of TiO₂. However, miR156 and miR162 were less sensitive to nano-TiO₂ exposure as they exhibited only less than a 2-fold change after 3 weeks of treatment. According to previous studies, miR156 regulates plant leaf development (Schwab et al. 2005; Zhang et al. 2006), and no study has reported that miR156 plays a function in stress response. In this study, we observed that miR156 expression is upregulated by 0.1 % nano-TiO₂ but

Fig. 3 Effect of nanoparticle TiO₂ on the expression of microRNAs (a) and two stress-related genes (b)



inhibited by 1.0 % nano-TiO₂. One potential reason for this change is that nano-TiO₂ inhibited tobacco seedling development and therefore the decrease in expression was due to a decrease in overall plant growth (Figs. 1 and 2).

TiO₂ nanoparticles affect the expression of stress-related genes in tobacco

In this study, we also observed that TiO₂ nanoparticle treatment affected the expression levels of stress-related genes. Both APX and ADH were significantly upregulated by both 0.1 and 1 % nano-TiO₂ concentrations after 3 weeks of exposure. ADH and APX showed relatively the same fold increase in expression in 0.1 % TiO₂ nanoparticle-exposed seedlings with ADH increasing 3.9-fold and APX increasing 3.6-fold. At a higher concentration (1.0 %), TiO₂ nanoparticles increased the expression of ADH to 5.73-fold, while the expression of APX was kept around a 3.5-fold change. This suggests that nanoparticle treatment also induces abnormal expression of stress-related genes, and plants may have similar mechanisms to handle both nanoparticle toxicity as well as other abiotic stresses.

Discussion

Nanoparticles are used in a variety of applications (paints, papers, manufactured products, etc.) and are interesting to study because their large surface area to volume ratio often times leads to an increase in reactivity (Gao and Zhang 2001). As nanotechnologies become increasingly used, nanoparticles will continue to be an environmental concern, and the phytotoxicity of these nanoparticles on terrestrial plants must be elucidated. In this study, we analyzed the effect of TiO₂ nanoparticles on the growth and development of tobacco seedlings. We found that as TiO₂ nanoparticle concentrations increased, the germination rates, leaf counts, root lengths, and overall biomasses of the tobacco seedlings decreased. We also found that TiO₂ nanoparticles influenced miRNA expression levels in tobacco.

The results of our study correspond to the results of other studies in which germination rates of ryegrass and corn were inhibited by Zn and ZnO nanoparticles (Lin and Xing 2007), and black cumin and wheat germination rates were inhibited by Zn²⁺ (El-Ghamery et al. 2003). Our results are slightly different from those found by Zheng et al. (2005), in which they found that low concentrations of nano-sized TiO₂ enhanced the germination rates of naturally aged spinach seeds (Zheng et al. 2005). While TiO₂ nanoparticles did not enhance the germination rate of the tobacco seeds, we did not observe a linear decrease in germination rate either, and germination rates rather decreased as TiO₂ nanoparticle concentrations reached 2.5 % and above. One potential reason is that Zheng et al. only used TiO₂ to treat spinach seeds, while we added

TiO₂ into the medium for longer exposure time. The seed coat has been shown to play a vital role in protecting the embryo from dangerous environmental factors (Wierzbicka and Obidzinska 1998). Lin and Xing (2007) offered the explanation that even though seeds are exposed to nanoparticles, the nanoparticles cannot invade the seed coat and effect germination (Lin and Xing 2007). Therefore, it is only after the seed begins to germinate that it comes into contact with the nanoparticles. In this study, we noticed a corresponding decrease in overall tobacco growth with an increase in TiO₂ nanoparticle concentration, suggesting that the nanoparticles have a negative impact on tobacco growth after seed germination has begun.

In this study, we also observed a decrease in average root length of tobacco seedlings as TiO₂ nanoparticle concentrations increased. Seedlings grown in 0.1 % nano-TiO₂ exhibited a decrease in average root growth by 48 % as compared to the control. An even greater inhibition of root growth was observed in 1 % nano-TiO₂ tobacco seedlings as the average root growth was reduced by 85 %. At 2.5 % nano-TiO₂, no root growth was observed. Therefore, we were able to show that TiO₂ nanoparticles have a negative impact on the development of tobacco roots.

The phytotoxicity of other metal oxide nanoparticles on the root growth of a variety of plant species has also been evaluated. Results have shown that nano-Al₂O₃ negatively impacts root development in corn (Lin and Xing 2007) and that ZnO nanoparticles damage epidermal and cortical cells in the roots of ryegrass (Lin and Xing 2008), leading to an overall decrease in plant growth. The mechanism by which TiO₂ nanoparticles affect root growth in tobacco still needs to be investigated; however, a recent study by Asli and Neumann (2009) showed that TiO₂ nanoparticles affected the water transport ability of maize roots. The authors were able to show that TiO₂ nanoparticles formed aggregates along the root cell wall that blocked the absorption of water, ultimately leading to reduced shoot and leaf development (Asli and Neumann 2009). Therefore, TiO₂ nanoparticles might inhibit tobacco root development by interfering with the pore on the root cell wall and reducing water uptake.

It should be noted that tobacco seedlings grown on 1 % nano-TiO₂ produced more than one root. If TiO₂ nanoparticles do adhere to the root cell walls of tobacco seedlings, the growth of more than one root would be necessary in order to increase water uptake and promote plant development.

Overall biomass of tobacco seedlings decreased as TiO₂ nanoparticle concentration increased. A 47 % decrease in biomass, as compared to the control, was observed for 0.1 % nano-TiO₂ seedlings. Interestingly, seedlings grown on 1 % nano-TiO₂ showed only a 37 % reduction in overall biomass. This result is surprising since a decrease in root development and an overall decline in plant health was noticed in these plants. The increase in biomass could be attributed to an intake

of TiO₂ nanoparticles. A recent study has shown that carbon-coated iron nanoparticles can be transported through the xylem in pumpkin plants (Corredor et al. 2009), and alternate studies have demonstrated that copper (Lee et al. 2008) along with Zn and ZnO (Lin and Xing 2008) nanoparticles can also be taken in by plant roots. It is possible that, over the 3-week course of this experiment, TiO₂ nanoparticles entered into the tobacco seedlings through pores in the root cell wall. This would cause the slight increase in biomass that was observed for tobacco seedlings exposed to 1 % nano-TiO₂.

As the TiO₂ nanoparticle concentration increased, a visible reduction in plant health occurred. Tobacco seedlings exposed to increasing levels of nano-TiO₂ exhibited yellowing, wilting, reduced leaf sizes and leaf counts, reduced root growth, and a decrease in shoots. In the case of tobacco seeds grown on 2.5 % nano-TiO₂, no roots were formed. The results from our study do not agree with those by Zheng et al. (2005), in which they concluded that nano-TiO₂ improved the growth of spinach plants (Zheng et al. 2005). Our results, however, do agree with others and appear to be symptoms of heavy metal toxicity (El-Ghamery et al. 2003; Lin and Xing 2008) and indicate cell death (Abraham 1997). Since TiO₂ nanoparticles have been shown to induce the formation of reactive oxygen species leading to oxidative DNA damage and lipid peroxidation (Falck et al. 2009; Federici et al. 2007; Trouiller et al. 2009), a future study is needed to determine the mechanisms of TiO₂ nanoparticle toxicity on tobacco. Alternate studies, as suggested by Murashov (2006), are also needed to determine if disassociation of the titanium ion is a cause of phytotoxicity rather than the TiO₂ nanoparticles themselves (Murashov 2006).

miRNAs are a newly discovered class of small regulatory RNAs (Bartel 2004) that have been shown to play a role in plant response to many environmental stresses such as drought (Zhao et al. 2007), salinity (Jia et al. 2009), and cold (Zhang et al. 2009). miRNAs have also been identified in plant response to heavy metals. For instance, Sunkar et al. (2006) have shown that miR398 targets two related superoxide dismutase genes in *Arabidopsis* and by downregulating this miRNA, the plant has a higher tolerance to copper and zinc metals (Sunkar et al. 2006). Other investigations have analyzed the effect of cadmium, aluminum, and mercury on miRNA expression levels in *Medicago truncatula* and rice and have found that miRNA expression levels are altered after exposure to heavy metals (Huang et al. 2009; Zhou et al. 2008b).

In this study, we analyzed changes in expression levels of 11 miRNAs in response to TiO₂ nanoparticles. We found that all 11 miRNAs were upregulated and, in comparison to the control plants, miR399b and miR395a in tobacco seedlings exposed to 1 % nano-TiO₂ exhibited the greatest fold changes of 143 and 285, respectively. The expression levels of two stress-related genes, ADH and APX, were also upregulated

after exposure to TiO₂ nanoparticles. Since miRNAs do play a significant role in negatively regulating gene expression, we can assume that the upregulation of these miRNAs is affecting the expression levels of many important developmental genes. This would explain, in part, the observed decrease in overall plant growth.

In conclusion, TiO₂ nanoparticles had a negative impact on the growth and development of 3-week-old tobacco seedlings. An overall reduction in germination rates, leaf counts, root lengths, and biomasses were observed in seedlings exposed to 0.1, 1, 2.5, and 5 % nano-TiO₂. This result could be due to the interference of TiO₂ clusters with the root cell wall, ultimately inhibiting water uptake, or the induction of reactive oxygen species leading to oxidative DNA damage and lipid peroxidation of the cell membrane in tobacco cells. More research needs to be performed in order to elucidate the mechanisms of TiO₂ nanoparticle toxicity and to determine if phytotoxicity is caused by dissociation of the titanium ion. As the manufacturing of TiO₂ continues to increase, TiO₂ nanoparticles will continue to be released into the environment. Studies have shown that TiO₂ nanoparticles can reach depths of between 40 and 370 cm in soil suspensions (Fang et al. 2009). Therefore, the phytotoxicity of TiO₂ on important crop plants, such as tobacco, must be investigated.

microRNA expression levels in tobacco were also altered after exposure to nano-TiO₂. In particular, miR399 and miR395 exhibited the greatest fold increases of 143 and 285, respectively. Our study analyzed changes in expression levels of 11 conserved miRNAs. In order to identify novel tobacco miRNAs that may play a role in plant tolerance to heavy metal stress, other experiments such as high throughput deep sequencing or direct cloning will be needed. We found that TiO₂ nanoparticles affected miRNA expression levels in tobacco and since they function as important gene regulators, miRNAs may play a vital role in tobacco response to heavy metal stress.

References

- Aarthi T, Madras G (2007) Photocatalytic degradation of rhodamine dyes with nano-TiO₂. *Ind Eng Chem Res* 46(1):7–14
- Abdel-Ghany SE, Pilon M (2008) MicroRNA-mediated systemic down-regulation of copper protein expression response to low copper availability in *Arabidopsis*. *J Biol Chem* 283:15932–15945
- Abraham S (1997) Studies on cytological changes induced by muriate of potash in *Allium cepa*. *Cytologia* 62(3):291–294
- Achard P, Herr A, Baulcombe DC, Harberd NP (2004) Modulation of floral development by a gibberellin-regulated microRNA. *Development* 131(14):3357–3365. doi:10.1242/dev.01206
- Adams LK, Lyon DY, Alvarez PJJ (2006) Comparative eco-toxicity of nanoscale TiO₂, SiO₂, and ZnO water suspensions. *Water Res* 40(19):3527–3532
- Asli S, Neumann PM (2009) Colloidal suspensions of clay or titanium dioxide nanoparticles can inhibit leaf growth and transpiration via

- physical effects on root water transport. *Plant Cell Environ* 32:577–584
- Aukerman MJ, Sakai H (2003) Regulation of flowering time and floral organ identity by a microRNA and its APETALA2-like target genes. *Plant Cell* 15(11):2730–2741. doi:10.1105/tpc.016238
- Baan R, Straif K, Grosse Y, Secretan B, Ghissassi FE, Coglianov V (2006) Carcinogenicity of carbon black, titanium dioxide, and talc. *Lancet Oncol* 7(4):295–296
- Bartel DP (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 116(2):281–297
- Boffetta P, Soutar A, Cherrie JW et al (2004) Mortality among workers employed in the titanium dioxide production industry in Europe. *Cancer Causes Control* 15(7):697–706
- Chen CZ, Li L, Lodish HF, Bartel DP (2004) MicroRNAs modulate hematopoietic lineage differentiation. *Science* 303(5654):83–86. doi:10.1126/science.1091903
- Chen CF, Ridzon DA, Broomer AJ et al (2005) Real-time quantification of microRNAs by stem-loop RT-PCR. *Nucleic Acids Res* 33(20):e179. doi:10.1093/nar/gni178
- Corredor E, Testillano PS, Coronado M-J et al (2009) Nanoparticle penetration and transport in living pumpkin plants: in situ subcellular identification. *BMC Plant Biol* 9:45. doi:10.1186/1471-2229-9-45
- Ding D, Zhang LF, Wang H, Liu ZJ, Zhang ZX, Zheng YL (2009) Differential expression of miRNAs in response to salt stress in maize roots. *Ann Bot* 103(1):29–38. doi:10.1093/aob/mcn205
- El-Ghamery AA, El-Kholy MA, El-Yousser MA (2003) Evaluation of cytological effects of Zn²⁺ in relation to germination and root growth of *Nigella sativa* L. and *Triticum aestivum* L. *Mutat Res* 537:29–41
- Falck G, Lindberg H, Suhonen S et al (2009) Genotoxic effects of nanosized and fine TiO₂. *Hum Exp Toxicol* 28:339–352
- Fang J, X-q S, Wen B, J-m L, Owens G (2009) Stability of titania nanoparticles in soil suspensions and transport in saturated homogeneous soil columns. *Environ Pollut* 157:1101–1109
- Federici G, Shaw BJ, Handy RD (2007) Toxicity of titanium dioxide nanoparticles to rainbow trout (*Oncorhynchus mykiss*): gill injury, oxidative stress, and other physiological effects. *Aquat Toxicol* 84(4):415–430
- Fryzek JP, Chadda B, Marano D et al (2003) A cohort mortality study among titanium dioxide manufacturing workers in the United States. *J Occup Environ Med* 45(4):400–409
- Fujii H, Chiou TJ, Lin SI, Aung K, Zhu JK (2005) A miRNA involved in phosphate-starvation response in *Arabidopsis*. *Curr Biol* 15(22):2038–2043. doi:10.1016/j.cub.2005.10.016
- Gao L, Zhang Q (2001) Effects of amorphous contents and particle size on the photocatalytic properties of TiO₂ nanoparticles. *Scr Mater* 44(8–9):1195–1198
- Hartmann NB, Von der Kammer F, Hofmann T, Baalousha M, Ottofuelling S, Baun A (2009) Algal testing of titanium dioxide nanoparticles—testing considerations, inhibitory effects and modification of cadmium bioavailability. *Toxicology*. doi:10.1016/j.tox.2009.08.008
- Higarashi MM, Jardim WF (2002) Remediation of pesticide contaminated soil using TiO₂ mediated by solar light. *Catal Today* 76(2–4):201–207
- Hsieh L-C, Lin S-I, Shih AC-C et al (2009) Unconverging small RNA-mediated responses to phosphate deficiency in *Arabidopsis* by deep sequencing. *Plant Physiol* 151:2120–2132
- Huang SQ, Peng J, Qiu CX, Yang ZM (2009) Heavy metal-regulated new microRNAs from rice. *J Inorg Biochem* 103(2):282–287. doi:10.1016/j.jinorgbio.2008.10.019
- Hund-Rinke K, Simon M (2006) Ecotoxic. Effect of photocatalytic active nanoparticles (TiO₂) on algae and daphnids. *Environ Sci Pollut Res* 13(4):225–232
- Jia XY, Wang W, Ren L et al (2009) Differential and dynamic regulation of miR398 in response to ABA and salt stress in *Populus tremula* and *Arabidopsis thaliana*. *Plant Mol Biol* 71(1–2):51–59
- Jones-Rhoades MW, Bartel DP (2004) Computational identification of plant MicroRNAs and their targets, including a stress-induced miRNA. *Mol Cell* 14(6):787–799
- Juarez MT, Kui JS, Thomas J, Heller BA, Timmermans MCP (2004) microRNA-mediated repression of rolled leaf1 specifies maize leaf polarity. *Nature* 428(6978):84–88. doi:10.1038/nature02363
- Kaegi R, Ulrich A, Sinnet B et al (2008) Synthetic TiO₂ nanoparticle emission from exterior facades into the aquatic environment. *Environ Pollut* 156(2):233–239
- Khraiwesh B, Zhu JK, Zhu JH (2012) Role of miRNAs and siRNAs in biotic and abiotic stress responses of plants. *Biochim Biophys Acta Genet Regul Mech* 1819(2):137–148. doi:10.1016/j.bbagr.2011.05.001
- Kiser MA, Westerhoff P, Benn T, Wang Y, Perez-Rivera J, Hristovski K (2009) Titanium nanomaterial removal and release from wastewater treatment plants. *Environ Sci Technol* 43(17):6757–6763
- Kubo M, Onodera R, Shibasaki-Kitakawa N, Tsumoto K, Yonemoto T (2005) Kinetics of ultrasonic disinfection of *Escherichia coli* in the presence of titanium dioxide particles. *Biotechnol Prog* 21(3):897–901
- Laufs P, Peaucelle A, Morin H, Traas J (2004) MicroRNA regulation of the CUC genes is required for boundary size control in *Arabidopsis* meristems. *Development* 131(17):4311–4322. doi:10.1242/dev.01320
- Lee W-M, An Y-J, Yoon H, Kweon H-S (2008) Toxicity and bioavailability of copper nanoparticles to the terrestrial plants mung bean (*Phaseolus radiatus*) and wheat (*Triticum aestivum*): plant agar test for water-insoluble nanoparticles environmental. *Toxicol Chem* 27(9):1915–1921
- Lewis BP, Burge CB, Bartel DP (2005) Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* 120(1):15–20. doi:10.1016/j.cell.2004.12.035
- Lin D, Xing B (2007) Phytotoxicity of nanoparticles: inhibition of seed germination and root growth. *Environ Pollut* 150:243–250
- Lin D, Xing B (2008) Root uptake and phytotoxicity of ZnO nanoparticles. *Environ Sci Technol* 42(15):5580–5585
- Lovern SB, Klaper R (2006) *Daphnia magna* mortality when exposed to titanium dioxide and fullerene (C₆₀) nanoparticles. *Environ Toxicol Chem* 25(4):1132–1137
- Lovern SB, Strickler JR, Klaper R (2007) Behavioral and physiological changes in *Daphnia magna* when exposed to nanoparticle suspensions (titanium dioxide, nano-C₆₀, and C₆₀HxC₇₀Hx). *Environ Sci Technol* 41(12):4465–4470
- Lubick N (2009) Hunting for engineered nanomaterials in the environment. *Environ Sci Technol* 43:6446–6447. doi:10.1021/es902174z
- Mallory AC, Dugas DV, Bartel DP, Bartel B (2004) MicroRNA regulation of NAC-domain targets is required for proper formation and separation of adjacent embryonic, vegetative, and floral organs. *Curr Biol* 14(12):1035–1046. doi:10.1016/j.cub.2004.06.022
- Mattigod SV, Fryxell GE, Alford K et al (2005) Functionalized TiO₂ nanoparticles for use for in situ anion immobilization. *Environ Sci Technol* 39(18):7306–7310
- Mlotshwa S, Yang ZY, Kim YJ, Chen XM (2006) Floral patterning defects induced by *Arabidopsis* APETALA2 and microRNA172 expression in *Nicotiana benthamiana*. *Plant Mol Biol* 61(4–5):781–793. doi:10.1007/s11103-006-0049-0
- Murashov V (2006) Comments on "Particle surface characteristics may play an important role in phytotoxicity of alumina nanoparticles" by Yang, L., Watts D.J., *Toxicology Letters*, 2005, 158, 122–132. *Toxicology Lett* 164(2):185–187
- Pan XP, Redding JE, Wiley PA, Wen L, McConnell JS, Zhang BH (2010) Mutagenicity evaluation of metal oxide nanoparticles by the bacterial reverse mutation assay. *Chemosphere* 79:113–116. doi:10.1016/j.chemosphere.2009.12.056
- Pott F, Roller M (2005) Carcinogenicity study with nineteen granular dusts in rats. *Eur J Oncol* 10(4):249–282

- Reeves JF, Davies SJ, Dodd NJF, Jha AN (2008) Hydroxyl radicals (.OH) are associated with titanium dioxide (TiO₂) nanoparticle-induced cytotoxicity and oxidative DNA damage in fish cells. *Mutat Res* 640(1–2):113–122
- Schwab R, Palatnik JF, Riester M, Schommer C, Schmid M, Weigel D (2005) Specific effects of MicroRNAs on the plant transcriptome. *Dev Cell* 8(4):517–527
- Sichel C, de Cara M, Tello J, Bianco J, Fernandez-Ibanez P (2007) Solar photocatalytic disinfection of agricultural pathogenic fungi: *Fusarium* species. *Appl Catal B Environ* 74(1–2):152–160
- Sunkar R, Zhu JK (2004) Novel and stress-regulated microRNAs and other small RNAs from *Arabidopsis*. *Plant Cell* 16(8):2001–2019. doi:10.1105/tpc.104.022830
- Sunkar R, Kapoor A, Zhu J-K (2006) Posttranscriptional induction of two Cu/Zn superoxide dismutase genes in *Arabidopsis* is mediated by downregulation of miR398 and important for oxidative stress tolerance. *Plant Cell* 18(8):2051–2065
- Trindade I, Capitaio C, Dalmay T, Fevereiro MP, Santos DM (2010) miR398 and miR408 are up-regulated in response to water deficit in *Medicago truncatula*. *Planta* 231(3):705–16
- Trouillier B, Reliene R, Westbrook A, Solaimani P, Schiestl RH (2009) Titanium dioxide nanoparticles induce DNA damage and genetic instability in vivo in mice. *Cancer Res* 69(22):8784–8789
- Vaucheret H (2006) Post-transcriptional small RNA pathways in plants: mechanisms and regulations. *Genes Dev* 20(7):759–771. doi:10.1101/gad.1410506
- Wang S, Chang L-Y, Wang Y-J, Wang Q, Yang C-H, Mei R-H (2009) Nanoparticles affect the survival of bacteria on leaf surfaces. *FEMS Microbiol Ecol* 68:182–191
- Wierzbicka M, Obidzinska J (1998) The effect of lead on seed imbibition and germination in different plant species. *Plant Sci* 137:155–171
- Xie XH, Lu J, Kulbokas EJ et al (2005) Systematic discovery of regulatory motifs in human promoters and 3' UTRs by comparison of several mammals. *Nature* 434(7031):338–345. doi:10.1038/nature03441
- Zhang BH, Pan XP, Cobb GP, Anderson TA (2006) Plant microRNA: a small regulatory molecule with big impact. *Dev Biol* 289(1):3–16. doi:10.1016/j.ydbio.2005.10.036
- Zhang J, Xu Y, Huan Q, Chong K (2009) Deep sequencing of *Brachypodium* small RNAs at the global genome level identifies microRNAs involved in cold stress response. *BMC Genomics* 10
- Zhao BT, Liang RQ, Ge LF et al (2007) Identification of drought-induced microRNAs in rice. *Biochem Biophys Res Commun* 354(2):585–590. doi:10.1016/j.bbrc.2007.01.022
- Zheng L, Hong F, Lu S, Liu C (2005) Effect of nano-TiO₂ on strength of naturally aged seeds and growth of spinach. *Biol Trace Elem Res* 104:83–91
- Zhou XF, Wang GD, Sutoh K, Zhu JK, Zhang WX (2008a) Identification of cold-inducible microRNAs in plants by transcriptome analysis. *Biochim Biophys Acta Genet Regul Mech* 1779(11):780–788. doi:10.1016/j.bbagr.2008.04.005
- Zhou ZS, Huang SQ, Yang ZM (2008b) Bioinformatic identification and expression analysis of new microRNAs from *Medicago truncatula*. *Biochem Biophys Res Commun* 374(3):538–542