



Absorption of zinc ions dissolved from zinc oxide nanoparticles in the tobacco callus improves plant productivity

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Abstract

Zinc oxide (ZnO) nanoparticles (NPs) are soluble in water and can release Zn^{2+} , an essential mineral that promotes the growth of plant cells. When ZnO NPs were administered to tobacco (*Nicotiana tabacum* cv. Samusun-NN) callus under white light irradiation, a concentration-dependent increase in weight was observed. Specifically, an increase in chlorophyll levels was triggered by blue light and induced by ZnO NPs. mRNA-seq analysis showed that during the early stages of tobacco callus exposure to ZnO NPs and white light irradiation, there was considerable fluctuation in the expression of genes related to salt stress. After 24 h, the expression of cellular component and growth-related genes also fluctuated. Analysis by RT-qPCR revealed that, after 1 day of ZnO NPs exposure, the expression levels of photosynthesis-related genes were enhanced. The Zn content of control-treated callus was 0.19 mg g⁻¹ dry weight, whereas that of callus cultured with the ZnO bulk particles (BPs), with a particle diameter of 2000 nm, was 2.59 mg g⁻¹ dry weight, and for callus cultured with ZnO NPs, with a particle diameter 34 nm, the Zn content was 3.37 mg g⁻¹ dry weight. These results indicate that ZnO particles supplied large amounts of Zn to the callus, suggesting that the smaller the particle size, the larger the surface area of particles dissolve zinc ions more efficiently and the more ions are supplied to tobacco callus cells, and resulting in an increase in plant productivity.

Key message

Under the light illumination, incubation of tobacco callus with zinc oxide nanoparticle dispersion resulted in supply of much zinc ions into cell, and induction of chlorophyll accumulation and cell proliferation.

Keywords Zn absorption · Chlorophyll increase · Photosynthesis

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Introduction

Nanoparticles (NPs) refer to particles 100 nm or less in size that exhibit catalytic, optical, and antibacterial properties different from those of bulk particles (BPs). Among metal oxide nanoparticles, CuO and ZnO have lower solubility than sulfate, and may elicit stress as ions acting on plant cells. When the Cu^{2+} ions released from CuO NPs exceeds the physiologically tolerable range, intracellular oxidative stress in algae is caused (Aruoja et al. 2009). On the other hand, positively influenced shoot organogenesis, shoot length and antioxidant activities of *Stevia* were reported when supplied up to 10 mg L⁻¹ (Javed et al. 2017). Lee et al. (2010) reported that, of four types of oxide NPs, exposure to ZnO NPs in the concentration range of 400–4000 mg L⁻¹ resulted in strong inhibition of seed germination and root elongation, as well as a decreased number in leaves, in

Arabidopsis thaliana. The inhibitory effect of ZnO NPs has been reported to be higher than that of ionic solutions without particles; additionally, since exposure to ZnO NPs leads to increased intracellular levels of zinc, an essential element for plants, ZnO NPs can promote plant growth at low concentrations. Indeed, ZnO NPs were shown to improve germination in cauliflower, tomato and cabbage, as well as subsequent seedling growth, at the 0.12–0.73 mg L⁻¹ concentration range, an improvement not seen with administration 0.37 mg L⁻¹ ZnO BPs (Singh et al. 2013). ZnO NPs do not inhibit the root elongation of rape and ryegrass at concentrations ≤ 10 mg L⁻¹, and the root elongation of radish at concentrations ≤ 20 mg L⁻¹ (Lin and Xing 2007). However, it is unknown why the inhibitory or promoting effects of ZnO NPs are not observed in particle-free ionic solution or with ZnO BP dispersion (BPD).

Plants grow through cell elongation and proliferation. Currently, few studies have reported on the effects of ZnO NPs on cell proliferation. Therefore, in this study, using the tobacco callus (a mass of cells undergoing active proliferation) as a model, we aimed to comprehensively evaluate and analyze the influence of ZnO NPs on cell proliferation. We also investigated changes in photosynthetic function occurring with the addition of ZnO NPs during callus cultivation. Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) functions in CO₂ fixation, and is comprised of a small subunit encoded by *rbcS* and a large subunit encoded by *rbcL*. In this study, we analyzed the influence of ZnO NPs on the photosynthetic function of the tobacco callus by comparing the chlorophyll content of cells and the expression levels of *rbcS* and *rbcL*. In order to understand in detail how gene expression is regulated by ZnO NPs, ZnO NP-dependent differential expression was comprehensively analyzed using mRNA-seq, and the genes with expression levels altered by ZnO NPs were then classified according to gene ontology.

Materials and methods

Callus induction and preparation of culture media

Callus induction was carried out on Murashige and Skoog (MS) medium (Murashige and Skoog 1962), pH 5.8, adjusted with a 0.1 M KOH solution. Two types of media were used, one for germination and containing no plant hormone, and the other for callus induction, containing 10⁻⁵ M NAA (1-naphthaleneacetic acid) and 10⁻⁶ M BA (benzyl adenine). Tobacco (*Nicotiana tabacum* cv. Samusun-NN) seeds were sterilized with 70% ethanol for 30 s, followed by 0.6% hypochlorous acid for 3–5 min, and washed three times with an excess of sterilized water. Tobacco seeds suspended in a 0.1% agar solution to prevent dryness were

sown on MS medium (pH 5.8) containing 1.5% agar, and grown in an incubator at 25 °C under continuous illumination (100 μmol photons m⁻² s⁻¹) for approximately 2 weeks. Grown tobacco leaves were cut with a scalpel, left on the callus-inducing solid medium, and again cultured in an incubator with the same conditions as above. Calli formed from 1 cm² leaf explant were cultured in liquid medium in the dark and subcultured to the same fresh liquid MS medium (pH 5.8) every 2 weeks.

Particle preparation

In this study, ZnO NPs (C. I. Kasei, Tokyo, Japan) with an approximate diameter of 34 nm and ZnO BPs with an approximate diameter of 2000 nm (Sakai Chemical Industry, Osaka, Japan) were used. 100 mg L⁻¹ ZnO particles were dispersed in the MS medium for 15 min with an ultrasonic washer (UA100; Kokusai Electric Inc., Tokyo, Japan) to obtain either a ZnO NP dispersion (NPD) or a ZnO BP dispersion (BPD). 25 and 50 mg L⁻¹ ZnO NPD was prepared by diluting 100 mg L⁻¹ ZnO with the MS medium. The ZnO NPD was centrifuged at 9300×g for 10 min, followed by ultrafiltration of the supernatant for 20 min using an ultrafiltration device (Vivaspin™ Turbo 15, Sartorius® Stedim Biotech, Gottingen, Germany) to obtain an ionic solution containing no particles. This solution was designated as Zn²⁺_(-NPs).

Exposure to ZnO particles

The callus was cultured for 2 weeks in a 50-mL Erlenmeyer flask supplemented with 12 mL of liquid MS medium (pH 5.8) with or without ZnO particle dispersions in the dark, or under white, red, or blue light LED irradiation (ISL-150 X 150-RR, CCS, Kyoto, Japan) (150 μmol photons m⁻² s⁻¹). After cultivation, the culture solution was filtered through a 1-mm diameter mesh to remove the culture medium and ZnO particles, and the fresh weight and amount of chlorophyll per gram fresh weight were measured. Average and standard error (S.E.) values were obtained from three independent experiments with three samples each, except in the red and blue light irradiation analysis, wherein the data were acquired from three samples. Statistical significance was determined with the Student's *t*-test comparing the control and NP treatment samples (*P* < 0.05). For chlorophyll extraction, callus and acetone of four times the fresh weight of callus was ground in a mortar, and the suspension was centrifuged. The absorption spectrum of the supernatant was measured, and the chlorophyll concentration (Chl *a* + *b*) was calculated using the following formula (Porra et al. 1989):

$$\text{Chl } a + b [\mu\text{g mL}^{-1}] = 17.76 \times (A_{646.6} - A_{750}) + 7.34 \times (A_{663.6} - A_{750})$$

For mRNA-seq analysis, tobacco callus was cultivated with or without ZnO NPD under white light for 1, 5, and 24 h, while cultivated with or without ZnO NPD under white light for 1 day for quantitative RT-PCR analysis.

Determination of absorbed zinc in callus

After culturing, the callus was filtered through a 1-mm diameter mesh and washed with distilled water. The washed callus was dehydrated in a convection oven (Yamato Scientific Co., Tokyo, Japan) at 60 °C for approximately 18 h, and then homogenized in a mortar. The crushed callus was dissolved in 60% nitric acid and heated at 100 °C to dryness. After drying and solidifying, the samples were redissolved in 1 M nitric acid and filtered through a 0.2- μ m filter. The filtrate was diluted tenfold with distilled water to produce a 0.1 M nitric acid solution. Zinc in the sample solution was quantified using a polarized Zeeman atomic absorption photometer ZA 3000 series (Hitachi High-Tech Science, Tokyo, Japan). In all the conditions, the difference between the zinc content per gram dry weight of control callus (i.e., the amount of zinc absorbed from the medium) and the zinc content per gram dry weight of treated callus was calculated.

Comprehensive gene expression analysis by mRNA-seq

Total RNA was extracted from 100 mg of callus cultured for 1, 5, and 24 h, using RNeasy Plant Mini Kit (QIAGEN, Hilden, Germany), and a library for next-generation sequencing was prepared from 4 μ g of total RNA using the TruSeq RNA Sample Prep Kit v 2 (Illumina, California, USA). The prepared library was comprehensively sequenced using MiSeq (Illumina, USA) and approximately 240 million reads were obtained from each sample. The obtained sequence reads were mapped to *N. tabacum* reference mRNA sequences (Sol Genomics Network) using TopHat (Trapnell et al. 2009), and expression levels

were quantified using Cufflinks (Trapnell et al. 2010). Functional annotation of the sequence reads obtained from *N. tabacum* calli was carried out by BLAST searches based on *A. thaliana* annotation information. In addition, differentially expressed genes (DEGs) between samples at each culture time were identified using Cuffdiff (Trapnell et al. 2010), with DEGs with a *q* value < 0.05 being identified as significant. Furthermore, to determine the gene function of the extracted DEGs, they were classified based on the gene ontology (GO) biological process categories, using DAVID Bioinformatics Resources (Dennis et al. 2003; Hosack et al. 2003).

Analysis of photosynthesis-related gene expression by qRT-PCR

Total RNA was extracted from approximately 100 mg of callus cultured for 1 day using the RNeasy Plant Mini Kit (QIAGEN, Germany). Genomic DNA was digested using recombinant DNase (Takara Bio Inc., Shiga, Japan), and cDNA was synthesized from the total RNA sample using Rever Tra Ace® kit (Toyobo, Osaka, Japan) with oligo dT primers. The cDNA was diluted tenfold and used as a template for quantitative real-time PCR (SYBR® Green Master Mix, Toyobo, Osaka, Japan) using the StepOne™ real-time PCR system (Thermo Fisher Sci., Tokyo, Japan) with gene-specific primers (Table 1). The following genes were targeted: the photosynthesis-related genes *rbcS* and *rbcL*; the key chlorophyll synthesis genes *HEMA1* (*glutamyl-tRNA reductase*), *CHLH* (*H subunit of Mg-chelatase*), *GUN4* (*genomes uncoupled 4*), *CHL27* (*membrane subunit of Mg-protoporphyrin IX monomethyl ester cyclase*), *PORC* (*protochlorophyllide oxidoreductase C*), and *CAO* (*chlorophyll a oxygenase*); and the transcription factor-coding genes *GLK1* (*golden2-like 1*) and *GLK2* (*golden2-like 2*). The results of the quantitative analysis are shown as abundance ratios relative to the *ACTIN* housekeeping gene.

Table 1 Sequences of gene-specific primers

Primer	Forward	Reverse
<i>HEMA1</i>	5'-ATTGGGCTCAGTATCCACAC-3'	5'-ATTGCAGAGCTCCCCAATAG-3'
<i>CHLH</i>	5'-AGTCAAGATGGTTGCAGAGC-3'	5'-GCAGCTTCACGAACATCAAC-3'
<i>GUN4</i>	5'-GAAAGCAGTGAAGTAGACCAG-3'	5'-AGTGGCAAATGTCTTCTGG-3'
<i>CHL27</i>	5'-AGGCTCTCTTGCAGGAATTC-3'	5'-AGAACTCAGCAGTGCAAGAC-3'
<i>PORC</i>	5'-TTGCAACTCCAGGTGTTACC-3'	5'-ATCCTGAAGAGGCTCCAGTAAC-3'
<i>CAO</i>	5'-AGCAGCAGAAGACGAAGAAC-3'	5'-TTGGGGCATGTTCTGCTTTC-3'
<i>GLK1</i>	5'-TGCCGCCATGTACAAAGTAG-3'	5'-AAGTACAAGGAGTTTGGG-3'
<i>GLK2</i>	5'-GCGGAAAGAGAGACATAAACCC-3'	5'-TAAAATGAGGCGGCACCATG-3'
<i>rbcS</i>	5'-AAGAGGGCGAAGAAGGCATAC-3'	5'-ACTGATGCACTGCACTTGAC-3'
<i>rbcL</i>	5'-AGCGTTACGTATGTCTGGTGA-3'	5'-ATACCGGACTTCGATCTTG-3'
<i>actin</i>	5'-GTGTTAGCCACACTGTCC-3'	5'-TCAGTCAAGTCACGACCAGC-3'

Results and discussion

Effect of ZnO NPs on callus proliferation

Table 2 shows the exposure concentrations of NPs that show toxicity, obtained from studies on the phytotoxicity of metal oxide NPs, indicating that toxicity is observed at a wide range of concentrations (10 to 2000 mg L⁻¹). In this study, the concentration-dependent effect of ZnO NPs on tobacco callus proliferation was analyzed. Figure 1 shows the fresh weight of tobacco callus cultured with 0–100 mg L⁻¹ ZnO NPD for 2 weeks under white light irradiation. After cultivation, the fresh weight of the control was 4.4 g, while for the callus cultured with the ZnO NPD at 25, 50, and 100 mg L⁻¹ it was 5.9, 6.9, and 6.6 g, respectively (Fig. 1). Above 25 mg L⁻¹, ZnO NPs significantly promoted callus growth, an effect that was even more pronounced with 100 mg L⁻¹ NPs. In contrast, when cultured with 100 mg L⁻¹ TiO₂ and SiO₂ NPDs, no differences in fresh weight were observed compared to the control (data not shown), although 10–200 mg L⁻¹ SiO₂ or 160–800 mg L⁻¹ TiO₂ NPs are known to be toxic to plant growth (Table 2). This suggests that among the metal oxide NPs tested, only ZnO NPs could activate photosynthetic growth in tobacco callus.

Enhancement of chlorophyll production by ZnO NPs under blue light

Figure 2 shows the callus chlorophyll content per gram fresh weight when irradiated with either blue or red light, and cultured with 100 mg L⁻¹ ZnO NPD for 2 weeks. Under red light irradiation, the chlorophyll content was 0.42 μg g⁻¹ fresh weight in the control, and 0.38 μg g⁻¹ with the ZnO NPD treatment. In contrast, under blue light irradiation, the chlorophyll content was 0.99 μg g⁻¹ and 6.12 μg g⁻¹ for the control and ZnO NPD treatments, respectively. These data show that ZnO NPs increased the production of chlorophyll by blue light, but not by red light. Plants have multiple

Table 2 Exposure concentrations of NPs that show toxicity, obtained from previous studies on the phytotoxicity of oxide NPs

NPs	Plant species	Concentration (mg L ⁻¹)	References
SiO ₂	<i>Gossypium hirsutum</i>	10–200	Le et al. (2014)
TiO ₂	<i>Allium cepa</i> <i>Nicotiana tabacum</i>	160–800	Ghosh et al. (2010)
ZnO	<i>Raphanus sativus</i> <i>Brassica napus</i> <i>Lolium perenne</i>	20–2000	Lin and Xing (2007)
	<i>Zea mays</i> <i>Oryza sativa</i>	25–2000	Yang et al. (2015)

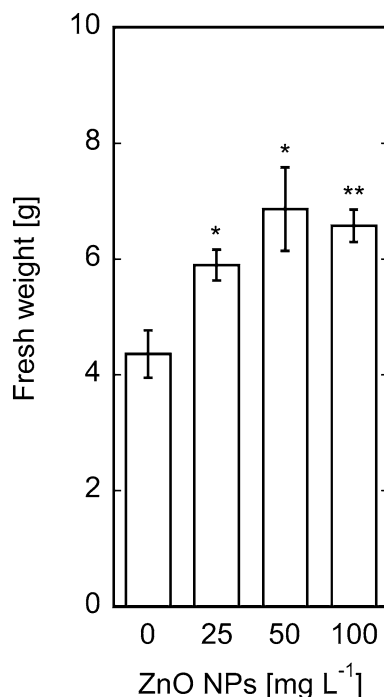


Fig. 1 Fresh weight of tobacco callus irradiated with white light and cultured with from 0 to 100 mg L⁻¹ ZnO NPD for 2 weeks. The data are shown as the mean ± S.E. of the results of three independent samples. * and ** Indicate a significant difference ($P < 0.05$ and $P < 0.01$, respectively), between ZnO NPD treatment and control treatment (0 mg L⁻¹) according to the Student's *t*-test

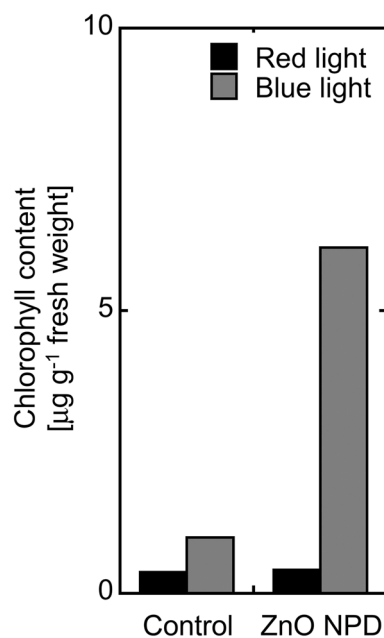


Fig. 2 Chlorophyll content per gram fresh weight of tobacco callus irradiated with either red (black bar) or blue light (gray bar) and cultured with 100 mg L⁻¹ ZnO NPD for 2 weeks. Chlorophyll content per gram fresh weight is shown as the average value of three independent samples

photoreceptors like phytochrome, phototropin, and cryptochrome that sense light and respond to changes in the light environment (Jiao et al. 2007). Cryptochrome and phytochrome are known to transmit light signals by regulating the transcription of photosynthesis-related genes in response to blue and red light, respectively (Ma et al. 2001). In this study, blue light, but not red light, appeared to be responsible for ZnO NP-dependent chlorophyll synthesis, indicating the involvement of cryptochrome. These results suggest that the increase in chlorophyll levels induced by ZnO NPs may be regulated via the blue light photoreceptor cryptochrome.

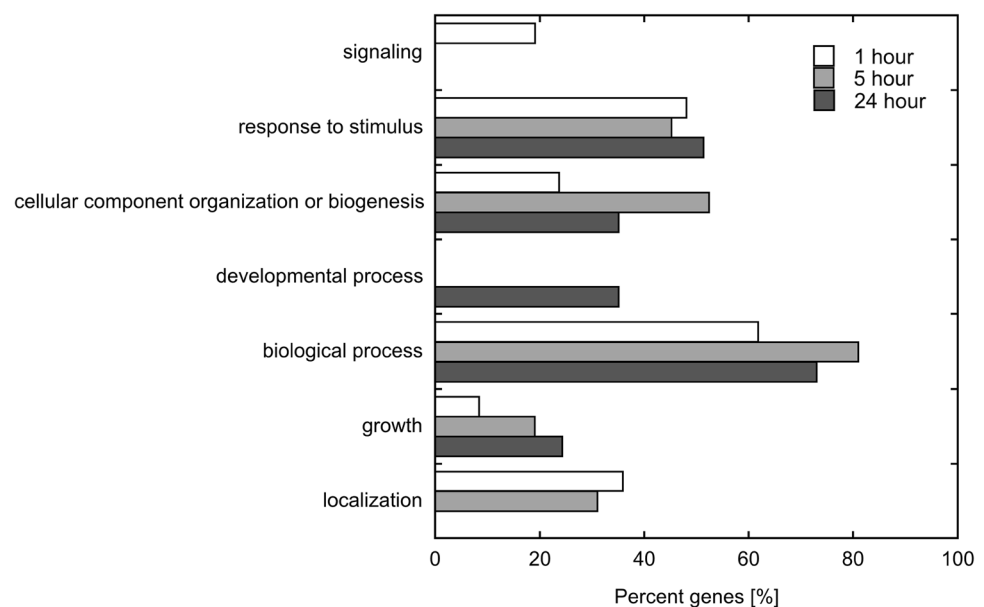
Comprehensive analysis of genes showing ZnO NP-dependent differential expression

To clarify the initial molecular responses of callus cultured in ZnO NPD, the callus gene expression profile was comprehensively analyzed using mRNA-seq. Compared to the control, the transcription of 131 genes was significantly altered by the ZnO NPD treatment after 1 h (q value < 0.05), of which 126 were upregulated, while 5 were downregulated. Five hours after exposure, 38 genes were upregulated, while 5 were downregulated; and after 24 h of ZnO NPD treatment, 9 genes were upregulated, while 29 genes were downregulated, suggesting that differential gene expression may have already occurred in the first hour of culture with ZnO NPD.

The results of classifying the DEGs into the biological process GO categories are shown in Fig. 3. The x -axis shows the percentage of genes classified as a specific GO term relative to the total number of DEGs. Approximately 19% of genes showing varied expression in the first hour of culture were classified as signaling (GO: 0023052); of these, the

transcription of genes encoding a number of SNF1—related protein kinase 2 (SnRK2) and 14-3-3 proteins was upregulated. Since SnRK2 is known to be involved in osmotic stress responses in *Arabidopsis* (Maszkowska et al. 2018), and 14-3-3 proteins are involved in a variety of metabolic processes in eukaryotes (Wilson et al. 2016), they may be involved in signal transduction in response to the ZnO NPD treatment. The genes upregulated in the first hour of treatment also included cellulose synthase and galacturonosyl transferase that are involved in cell wall synthesis. The expression of a number of genes coding for proteins of the RAB GTPase family, which have been shown to traffic cell wall polymers in *Arabidopsis* (Lunn et al. 2013), were also upregulated, suggesting the induction of cell wall modification. Furthermore, plasma membrane intrinsic proteins (aquaporins) were upregulated after 1 and 5 h of treatment, but not after 24 h. It has been shown that treating *A. thaliana* with 4 mg L^{-1} ZnO NPD for 7 days resulted in reduced levels of aquaporin transcripts (Landa et al. 2015), suggesting that aquaporin expression is induced in the early stages of ZnO NPD exposure, but is then subsequently suppressed. Of the genes upregulated after 5 h of culture, 58% were classified as cellular component organization or biogenesis (GO: 0071840), and include expansin-, histone-, and aquaporin-coding genes; in contrast, the expression levels of genes coding for amino acid permeases declined. At 24 h after culture, cell wall-loosening expansin-coding genes were upregulated, while the expression of the sucrose synthase- and phenylalanine ammonia lyase-coding genes that are involved in the biosynthesis of phenylpropanoid, a lignin monomer, was downregulated. In addition, there was a decline in the expression levels of genes coding for glutamate decarboxylase (GAD), peroxidase, alcohol dehydrogenase (ADH),

Fig. 3 Classifying the DEGs into GO categories for tobacco callus cultured with 100 mg L^{-1} ZnO NPD for 1, 5, and 24 h. The x -axis shows the percentage of the total number of DEGs classified as a specific GO term



and pyruvate decarboxylase (PDC). In many plant species, GAD is involved in the biosynthesis of γ -aminobutyric acid (GABA) under both biotic and abiotic stresses (Podlesáková et al. 2019). Peroxidase, ADH, and PDC have been shown to be involved in the response to hypoxia and other abiotic and biotic stresses in *Arabidopsis* (Ismond et al. 2003; Yang et al. 2011; Shi et al. 2017). In the early stages of ZnO NPs exposure, tobacco callus cells showed a stress response, in particular an osmotic stress-like response, although this was relieved and expression of cell growth-related factors was induced over 24 h.

Salt stress is accompanied by osmotic stress, where an extracellular change in solute concentration results in an altered state or in altered activity of the cells. The increase in the number of ions and the osmotic potential of the exposure solution could have led to the increased expression of salt stress- and osmotic stress-responsive genes in the tobacco callus. When *A. thaliana* plants were exposed to a ZnO NPs, BPs, or ZnSO₄ solution for 7 days, salt stress- and osmotic stress-responsive genes were also upregulated (Landa et al. 2015). The transcription of some expansin-coding genes was upregulated in 10-day-old *A. thaliana* plants subjected to 200 mM NaCl for 24 h, while other expansin-coding genes were downregulated. In this study, exposing tobacco callus to ZnO NPD for 24 h resulted only in the increased expression of expansin-coding genes. In addition, the transcription of four GAD-coding genes was downregulated in ZnO NPD-treated tobacco callus, while only one GAD gene was downregulated in NaCl-treated *Arabidopsis*. In this study, ZnO NPD did not inhibit tobacco callus growth, but instead promoted it. This suggests that tobacco callus cultured with ZnO NPD adapts to the stress environment by regulating the expression of genes involved in cellular organization and biosynthesis.

Increase in the expression of *rbcS* and *rbcL* induced by ZnO NPs

mRNA-seq analyses indicated that the expression levels of genes involved in chlorophyll biosynthesis or CO₂ fixation did not fluctuate significantly by ZnO NP treatment. To verify the transcription levels of these genes, quantitative real-time PCR was performed for tobacco callus cultured with 100 mg L⁻¹ ZnO NPD for 1 day under white light. The enzyme RuBisCO is composed of a small (RbcS) and a large (RbcL) subunit and catalyzes the first major step in the conversion of CO₂ into carbohydrates during photosynthesis. The relative expression levels of *rbcS* and *rbcL* on day 1 of culture were 9.5 and 3.0, respectively, compared to control, indicating that the expression of RuBisCO subunit-coding genes clearly increased with ZnO NPD treatment (Table 3). The increase in the fresh weight of callus may

Table 3 Expression analysis of photosynthetic-related genes in tobacco callus cultured with 100 mg L⁻¹ ZnO NPD on day 1 of culture under white light. At each day, the expression level in the control tobacco callus was taken as 1. Data are the result of 3 independent samples \pm standard error

	<i>rbcS</i>	<i>rbcL</i>
Relative expression [-]	9.5 \pm 2.1	3.0 \pm 0.95

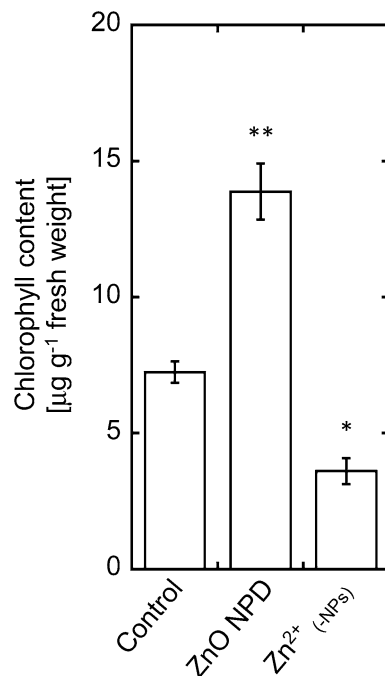
have resulted from increased production of chlorophyll that absorbs light energy in photosynthesis, and increased expression of photosynthesis-related genes. It has been reported that the overexpression of *OsGLK1*, encoding a GARP (GOLDEN2, ARR-B, Psr1) transcription factor, in non-green rice callus resulted in the increased expression of photosynthesis-related genes encoded in the both nuclear and plastid genome (Nakamura et al. 2009). Moreover, the chloroplasts in callus overexpressing *OsGLK1* developed grana stacks in a light-dependent manner (Nakamura et al. 2009). The tobacco callus used in this study was also not green regardless of light irradiation; however, the callus turned green only when it was cultured with ZnO NPD under light; this suggests that ZnO NPD treatment may have led to the development of chloroplasts with increased photosynthetic capacity, in a light-dependent manner, compared to chloroplasts from the control treatment. It was also reported that blue light is more effective than red for chloroplast development in roots (Usami et al. 2004), consistent with our data showing a significant increase in chlorophyll content in tobacco callus cultured with ZnO NPs under blue light irradiation.

Increase in the expression of key chlorophyll biosynthetic and GLK genes induced by ZnO NPs

Table 4 shows the results of the expression analysis of key chlorophyll biosynthetic genes and *GLK* transcription factor genes in tobacco callus cultured with 100 mg L⁻¹ ZnO NPD, on day 1 of culture. *GLKs* genes are known to encode transcription factors with a strong inducible effect on key chlorophyll biosynthetic genes (Waters et al. 2009). The relative expression levels of key genes involved in chlorophyll biosynthesis, namely *HEMA1*, *CHLH*, *GUN4*, *CHL27*, *PORC*, and *CAO*, were increased by 3.0-, 3.9-, 3.3-, 3.7-, 5.1-, and 1.9-fold, respectively. The relative expression levels of *GLK1* and *GLK2* were increased by 4.2- and 5.1-fold, respectively. The results from callus cultured with ZnO NPD suggest that the increased expression of genes key for chlorophyll biosynthesis was induced by the increased expression of *GLK*.

Table 4 Expression analysis of key chlorophyll biosynthetic genes and *GLK* transcription factor genes in tobacco callus cultured with 100 mg L⁻¹ ZnO NPd under white light on day 1 of culture. For each

	<i>HEMA1</i>	<i>CHLH</i>	<i>GUN4</i>	<i>CHL27</i>	<i>PORC</i>	<i>CAO</i>	<i>GLK1</i>	<i>GLK2</i>
Relative expression [-]	3.0±0.85	3.9±1.4	3.3±1.4	3.7±1.4	5.1±2.8	1.9±0.5	4.2±1.1	5.1±2.3

**Fig. 4** Chlorophyll content per gram fresh weight of tobacco callus cultured for 2 weeks with 100 mg L⁻¹ ZnO NPd or Zn²⁺_(-NPs) solutions. The data are shown as the mean±S.E. of results from three independent samples. * and ** Indicate a significant difference ($P < 0.05$ and $P < 0.01$, respectively), between ZnO NPd or Zn²⁺_(-NPs) treatment and the control according to the Student's *t*-test

Comparison of chlorophyll content in calli cultured in particle-free ion or NPd solutions

The chlorophyll content per gram of fresh callus weight was analyzed after culturing the callus for 2 weeks with 100 mg L⁻¹ ZnO NPd or Zn²⁺_(-NPs) solutions under white light (Fig. 4). The chlorophyll content was 13.9 µg g⁻¹ fresh weight when callus was grown in ZnO NPd-containing particles, and was higher than in the control or when grown in a Zn²⁺_(-NPs) solution. The chlorophyll content in callus tissue may have increased only when it was cultured with ZnO NPd-containing particles. An oxide particle can be either positively or negatively charged depending on the pH of the solution, and its surface is hydrated to form -OH groups. It becomes positively charged as the pH decreases, and negatively charged as the pH increases. It was reported that a ZnO particle has a surface charge of zero at pH 9.3, and is

culture day, the expression level in the control callus was taken as 1. Data are the result of 3 independent samples±standard error

Table 5 Zinc content per gram dry weight of tobacco callus cultured for 2 weeks in 100 mg L⁻¹ dispersions of different ZnO particle sizes under white light. Zinc content per gram dry weight is the average value of three independent samples

	Control	ZnO NPd	ZnO BPD
The zinc content per dry weight [mg dry-g ⁻¹]	0.19	3.37	2.59

positively charged at pH 6.0 (Zhai et al. 2010). In this study, the pH of the culture solution was adjusted to 5.8, therefore the ZnO NPs were positively charged. In contrast, the surface of the plant cell was likely negatively charged since the plant cell wall is composed largely of cellulose containing many carboxyl groups. Indeed, ZnO NPs have been shown to adhere to cell surfaces in plants and algae (Lin and Xing 2008; Aruoja et al. 2009; Jiang et al. 2009; Mahajan et al. 2011). It was also reported that when ZnO NPs adhere to algae, electrostatic attractions between the cell walls, membranes, and ZnO NPs promote uptake of released Zn²⁺ and induce membrane depolarization (Chen et al. 2012). Considering the mRNA-seq results in this study, Zn²⁺ released from particles attached to the cell surface may be more readily absorbed into the cell.

Chlorophyll content of callus cultured with particle dispersions of different sizes

If the NPs adhere to the cell surface and Zn²⁺ that is released locally on the cell surface is absorbed into the cells, then the smaller the particle size, the larger the area adjacent to the cell surface, leading to more Zn²⁺ absorption into the cell. Table 5 shows the zinc content per gram dry weight of callus cultured for 2 weeks under white light in 100 mg L⁻¹ dispersions of different ZnO particle sizes. Control callus contained 0.19 mg Zn²⁺ g⁻¹ dry weight, whereas those cultured with ZnO BPD contained 2.59 mg Zn²⁺ g⁻¹ dry weight and those cultured with ZnO NPd contained 3.37 mg Zn²⁺ g⁻¹ dry weight. Therefore, Zn²⁺ dissolved from NPs attached to the cell surface was better absorbed into the callus than Zn²⁺ dissolved in the MS medium (29.91 µg L⁻¹), although ZnO NPd was likely to supply more Zn²⁺ than BPD.

The chlorophyll content per gram fresh weight of callus cultured for 2 weeks under white light with 100 mg L⁻¹ ZnO NPd or BPD is shown in Fig. 5. The chlorophyll content in

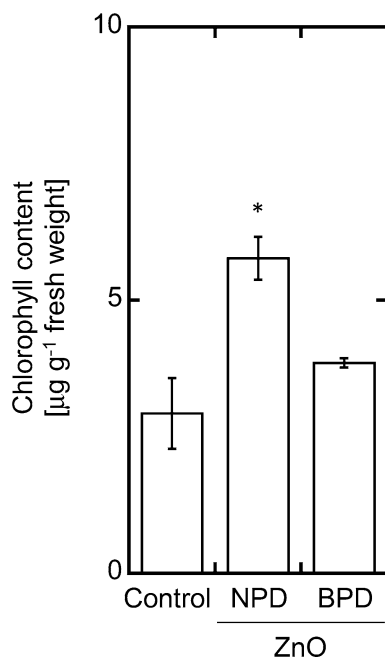


Fig. 5 Chlorophyll content per gram fresh weight of tobacco callus cultured for 2 weeks with 100 mg L⁻¹ ZnO NPD or BPD. The data are shown as the mean ± S.E. of results from three independent samples. * Indicates significant differences ($P < 0.05$) between ZnO NPD and the control according to the Student's *t*-test

the control callus was 2.93 µg g⁻¹ fresh weight, whereas it was 5.77 and 3.85 µg g⁻¹ fresh weight when callus was cultured in ZnO NPD and ZnO BPD, respectively. Therefore, the chlorophyll content of callus cultured with ZnO NPD was approximately twofold higher than for the control callus.

Zinc is an important constituent of numerous enzymes and plays a role in stabilizing the structure of proteins and cell membranes (Broadley et al. 2007). It was reported that, under zinc deficiency, the chlorophyll contents decrease, the microstructure of the chloroplast is destroyed, and the activities of the electron transfer and photosynthetic systems decrease (Aravind and Prasad 2004). In the present study, the biosynthesis of various proteins necessary for chloroplast development was likely induced by the high rate of zinc absorption from ZnO NPs by the cells. In addition, we suggest that by administering NPs with smaller particle size than those of BPs during culture, zinc ions were more readily absorbed into the cells, resulting in increased callus growth.

Zinc is an essential element for the growth of plants. Intracellular ion concentrations are higher than those in the soil; therefore, ion uptake from root cells is energy-dependent. In contrast, when a large amount of fertilizer is administered, the root cells are subjected to osmotic stress, resulting in water deficiency and inhibition of essential ion uptake by extracellular ion accumulation. The results of the present study suggest that administering strongly

adhering soluble NPs as fertilizer may lead to the gradual liberation of ions near the cell surface, which can then be rapidly absorbed into the cell. In recent years, next-generation biofuels derived from photosynthetic microalgae have attracted increasing attention because these biofuels are more productive than those derived from plants (Sivakumar et al. 2012; Salama et al. 2018; Nhat et al. 2018). Therefore, improved biofuel production efficiency could be achieved by administering soluble oxide NPs not only to plants, but also to microalgae.

Conclusions

Under light conditions, culturing tobacco callus with ZnO NPD led to an increase in photosynthetic processes like the promotion of chlorophyll biosynthesis and increased expression of photosynthesis-related genes, as well as to the promotion of cell proliferation. Exposure of tobacco callus to ZnO NPD accumulated more chlorophyll in the cell, compared with Zn²⁺_(-NPs) exposure. Since zinc ions are necessary for maintaining the structure of zinc finger-type transcription factors and also act as cofactors for chlorophyll synthase, tissues that actively proliferate require zinc ions. The smaller the particle size of ZnO particles, the more zinc ions were absorbed in tobacco callus cells, resulting in an increase in chlorophyll content in the cells.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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