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Was improvement of spinach growth by nano-TiO_2 treatment related to the changes of Rubisco activase?

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Abstract Characterized by a photo—catalysis property, nano-anatase TiO_2 is closely related to photosynthesis of spinach. It could not only improve light absorbance, transformation from light energy to electron energy and active chemical energy, but also promote the activity of Rubiso activase of spinach. However, the relation between the activity of Rubiso activase and the growth of spinach promoted by nano-anatase TiO_2 treatment remains largely unclear. In this study, we find that the amount and the activity of Rubiso activase are obviously increased by nano-anatase TiO_2 treatment, which led to the great promotion of Rubsico carboxylation and the high rate of photosynthesis, thus improving of spinach growth. The significant enhancement of Rubiso activase activity of nano-anatase TiO_2 treated spinach is also accompanied by conformational changes as determined by spectroscopic analysis. But bulk TiO_2 effect is not as significant as nano-anatase TiO_2, as the grain size of nano-anatase TiO_2 (5 nm) is much smaller than that of bulk TiO_2, which entered spinach cell more easily.

Keywords Nano-anatase TiO_2 · Spinach · Rubsico activase · Spectral property

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Introduction

Nano materials (NM) and nano technology have been widely applied in world. Recently, nano compound fertilizer has also been applied in agriculture. The effect of NM on plant growth to date was also reported (Wang et al. 1999). However, few studies have focused on the mechanisms of NM effect on high plant. As shown by a photo—catalyzed characteristic, nano-TiO_2 under light could cause oxidation-reduction reaction (Crabtree 1998). Considering the fact, we speculate that nano-TiO_2 might interfere with photosynthesis of plants. Our previous results showed that nano-TiO_2 treatments could markedly promote aged seeds vigor and chlorophyll biosynthesis of spinach, particularly, the ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) activity and the photosynthesis efficiency. The nano-TiO_2 treatments also have obvious effects on the improvement of growth and development in spinach, however bulk TiO_2 (non-nano-TiO_2) treatments have only little effects (Zheng et al. 2005). The studies on improving photosynthesis of spinach suggested that nano-anatase TiO_2 could increase light absorbance, accelerate transport and transformation of the light energy, protect chloroplasts from ageing and prolong photosynthetic time of chloroplasts (Hong et al. 2005a, b, c). Our recent results proved that the complex of Rubsico and Rubisco activase could be induced in nano-anatase TiO_2 treated spinach, which promoted Rubsico carboxylation

and increased the rate of photosynthetic carbon reaction (Gao et al. 2006).

Rubisco catalyzes the carboxylation and oxygenation of ribulose-1, 5-bisphosphate(RuBP), the first committed steps in the competitive metabolic pathways of photorespiration and photosynthetic CO_2 fixation in higher plants (Hong et al. 2005c; Spreitzer 1999). Recently, physiologists and biochemists showed much interest in the activation mechanism, structure, function and molecular biology of Rubisco (Keegstra et al. 1989; Chua and Schmidt 1979). The regulation of Rubisco activation in vivo depends on Rubisco activase (R-A), a chloroplastic enzyme of nuclear encoding. The activation of Rubisco driven by Rubsico activase is a photophosphorylation hydrolysis-dependent action, followed by an immediate binding of CO_2 and Mg^{2+} at the physiological level and the formation of an enzyme-CO_2-Mg (ECM) complex, thus gaining a maximal activation speed. Rubisco activase releases the inhibition from RuBP on Rubisco, and is possibly a new member of the molecular chaperone family (Portis et al. 1986, 1995; Jimenez et al. 1995; Han et al. 2000). Robison and Lan et al. (Robison et al. 1988; Lan and Meott 1991) thought that the activation mechanism of Rubsico activase on Rubisco involved in a formation of Rubisco and Rubsico activase intermediate complex, which could change the conformation of Rubisco, cause the Rubisco dissociation from RuBP and easily bind CO_2 to the activated sites. Then the carbamylation of Rubisco would be accelerated (Robison et al. 1988; Lan and Meott 1991; Robert et al. 2003), which led to the promotion of Rubsico carboxylation. Therefore, the activity of Rubisco activase is one of key factors for regulating Rubisco activity.

As we know, about 50% of the total soluble protein in spinach leaves are Rubisco, and the Rubisco activase takes only 2% (Robison et al. 1988), thus the activity of Rubisco activase is the most important event in promoting Rubsico carboxylation and formatting the complex of Rubsico and Rubisco activase. Nano-anatase TiO_2 treatment can promote photosynthetic carbon assimilation by inducing the formation of a Rubisco-R-A complex (Gao et al. 2006). However, the reason of the Rubisco activase activity promoted by nano-anatase TiO_2 treatment and the relation between the Rubisco activase activity and the growth of spinach are still unclear. In this study, we proved that the amount and the activity Rubisco activase are obviously increased in nano-anatase TiO_2 treated spinach, which enable the great promotion of Rubsico carboxylation and the high rate of photosynthesis, thus leading to the improvement of spinach growth. As shown by spectroscopic analysis, the significant enhancement of Rubisco activase (R-A) activity of nano-anatase TiO_2 treated spinach is attributed to conformational changes.

Materials and methods

Material treatment and culture

Experimental material was *Spinacia oleracea*. The seeds were purchased in a local seed company. Nano-anatase TiO_2 was prepared by Yang Ping's laboratory via controlled hydrolysis of titanium tetrabutoxide. The details of the synthesis can be found elsewhere (Yang et al. 2002). The average grain size calculated from broadening of the (101) XRD peak of anatase using Scherrer's equation was ca 5 nm. Spinach seeds were soaked with 0.03% nano-anatase TiO_2, 0.03% bulk TiO_2 suspension for 48 h at 10°C under light, and with deionized water for control, respectively. Then, the seeds were planted in an experimental flowerpot. Spinach seedlings in the stage of two leaves were sprayed with 0.03% nano-anatase TiO_2 and bulk TiO_2 suspension once a week until it has eight leaves.

Growth measurement of spinach

Fresh weight and dry weight of single plants were measured in the stage of eight leaves. And the chlorophyll contents were determined according to Arnon's method (Arnon 1949), and photosynthetic rate was detected with a portable photosynthesis detector of CI-301PS (CID Co., USA). Carboxylase activities of crud Rubisco and activities of crud Rubisco activase were measured with the method of Wang and Li (1980) (Fig. 1).

Purification of Rubisco, Rubisco activase and Assay of enzyme activity

Rubisco and Rubisco activase were purified according to the procedures reported by Sugiyama et al.

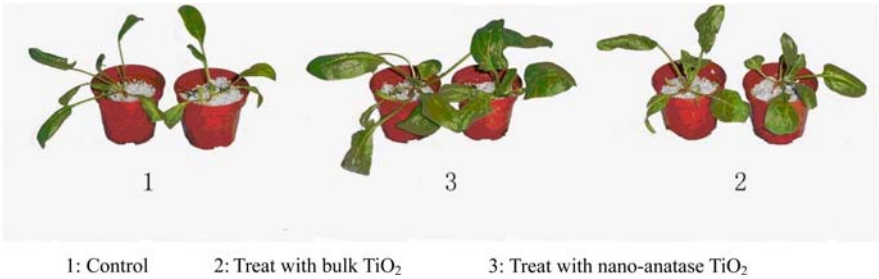

Fig. 1 Effect of nano-anatase TiO_2 on spinach growth. The spinach was cultured as described in 'Materials and methods'. The picture was taken after four weeks cultivation

(Sugiyama et al. 1968) and Salvucci et al. (Salvucci and Klein 1994), respectively. The concentration of protein was determined by the method of Lowry (Lowry et al. 1951). Assay of Rubisco carboxylase activity was performed in the same way as Li's (Tang et al. 1997). The activity of Rubisco activase was detected according to previous work (Jimenez et al.1995).

Polyacrylamide gel electrophoresis and Western blot

Denaturing (SDS-PAGE) polyacrylamide gel electrophoresis was conducted as described by Laemmli (1970). In this work, 15% (w/v) resolving gel coupled to a 5% (w/v) stacking gel were utilized. Polyclonal antibodies, recognizing Rubisco and Rubisco activase, were raised in New Zealand White rabbits as described by Sambrook and Fritsch (Sambrook and Fritsch 1989). Western-blot was carried out according to To's methods (To et al. 1996).

UV–Vis absorption spectrum of purified Rubisco activase

The absorption spectra of the purified Rubisco activase were measured at 273 K with a dual-beam spectrophotometer (UV-3010, Hitachi Co., Japan).

Fluorescence emission spectrum of purified Rubisco activase

The fluorescence emission spectra of the purified Rubisco activase were recorded at 273 K and 300–500 nm on a F-4500 fluorometer (UV-3010, Hitachi Co., Japan). Excitation was at 285 nm. The concentration of Rubisco was 0.06 mg/ml

Assay of circular dichroism (CD) spectrum of the purified enzyme

CD spectrum was detected at room temperature on JASCO-J-810 spectropolarimeter with a quartz sample cell of an optical path length of 1 cm. The concentration of Rubisco was 0.2 mg/ml. Molecular ellipticities $[\theta]$ in deg $cm^2.dmol^{-1}$ were calculated using mean residue weight of 114.2. The secondary structure indexes, α-helix, β-sheet, β-turn and random coil of enzyme samples were determined by using Perczel's method (Perczel et al. 1992).

Results and discussion

Effect of nano-anatase TiO_2 on growth of spinach

The growth of spinach was greatly improved by nano-anatase TiO_2 treatment. As shown in Table 1, the single fresh weight and dry weight of spinach with nano-anatase TiO_2 treatment were increased by 60.21% and 70.32%, respectively. The chlorophyll contents were enhanced by 17.23%; and the net photosynthetic rate was promoted by 28.82% as compared with the control, while the bulk TiO_2 treated spinach had no such significant changes. These results indicated that, by improving the growth of spinach, accelerating the synthesis of chlorophyll and promoting the photosynthesis rate, nano-anatase TiO_2 could significantly increase the dry weight of spinach, demonstrating that nano-anatase TiO_2 treatment could promote the accumulation of cellular compounds of spinach.

The carboxylase activity of crud Rubisco and the activity of crud Rubisco activase in bulk TiO_2 treated spinach were nearly the same as the control, while

Table 1 Effect of 0.25% nano-TiO_2 on growth of spinach

Indexes	Control	Bulk-TiO_2	Nano-anatase TiO_2
Single plant fresh weight (g)	3.43 ± 0.18[a]	3.56 ± 0.16[a]	5.49 ± 0.21[b]
Single plant dry weight (g)	0.61 ± 0.07[a]	0.63 ± 0.08[a]	1.04 ± 0.04[b]
Chlorophyll content (mg g^{-1}FW)	1.39 ± 0.09[a]	1.42 ± 0.07[a]	1.63 ± 0.10[b]
Net photo-synthetic rate (μmol CO_2 m^{-2} s^{-1})	21.95 ± 1.08[a]	22.04 ± 1.12[a]	28.28 ± 1.21[b]
Rubisco carboxylase activity (μmol CO_2 mg^{-1}·protein min^{-1})	0.53 ± 0.01[a]	0.56 ± 0.02[a]	0.95 ± 0.03[b]
Rubisco activase activity (μmol CO_2 mg^{-1} protein min^{-1})	0.25 ± 0.01[a]	0.28 ± 0.03[a]	0.60 ± 0.02[b]

The indexes listed above were determined by the assays described in 'Materials and methods'. The data presented are an average of the recordings from five independent experiments. a and b indicates that the differences of values followed by different letters in the same line are significant at $P = 0.05$ level (t test)

nano-anatase TiO_2 treatment resulted in significant improvement, by up to 1.9, 2.5 times to the control, respectively, which might be the reason the enhanced photosynthetic rate and accumulation of organic substances of spinach.

Purification, assay of electrophoresis and Western-blot of Rubisco activase

In order to make sure whether or not the nano-anatase TiO_2 treatment has an effect on the contents of Rubsico activase, we purified Rubisco activase from each 50 g spinach leaves. The results showed that the contents of Rubisco activase from the nano-anatase TiO_2 treated spinach was increased by 42% compared with the control, while bulk TiO_2 treatments resulted in the 5% improvement. The above results were further confirmed by SDS-PAGE electrophoresis and Western-blot, in this course, 1% (5 μl) of each purified protein was assayed and the results were shown in Fig. 2a and b. Consistent with earlier results, two bands at 41 and 45 kDa were observed (see in Fig. 2a), which respectively attributed to two subunits of Rubisco activase (Liu et al. 2006; Hong et al.2005d). The bands of "Rubisco activase" purified from the nano-anatase TiO_2-treated spinach (Lane 3 in Fig. 2a, b) are much broader compared with the control (Lane 1 in Fig. 2a, b), while the strands of bulk TiO_2-treated spinach were nearly the same as the control (Lane 2 in Fig. 2a, b).

Therefore, the nano-anatase TiO_2 treatment increased the amounts of Rubisco activase. High

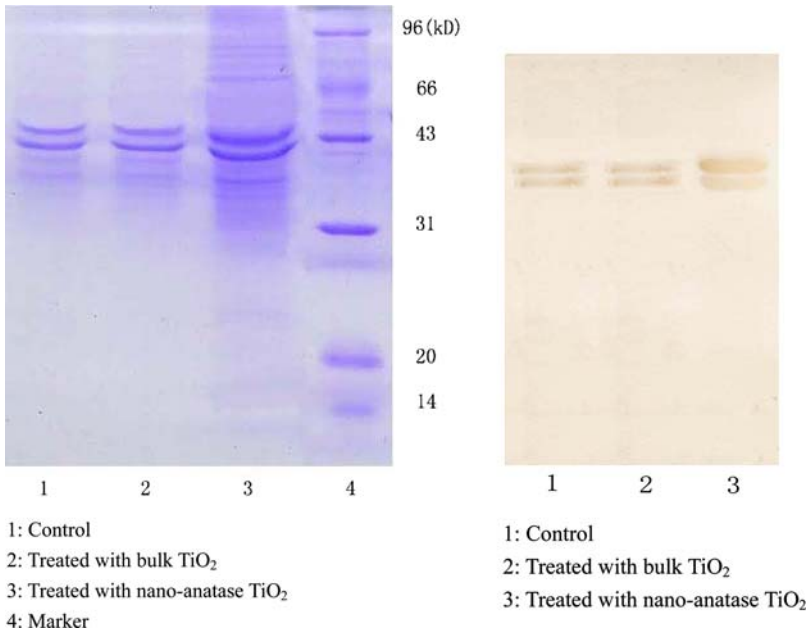

Fig. 2 (**a**) and (**b**) Western-blot of SDS-PAGE. Purified protein, control spinach Rubisco activase (lane 1), normal TiO_2 treated spinach Rubisco activase (lane 2), and nano- anatase TiO_2 treated spinach Rubisco activase (lane 3) were resolved by SDS-PAGE (5 μl each). Gels were either stained with Coomassie blue (Fig. 3) or transferred to nitrocellulose membranes for Western-blot analysis and revealed with antibodies raised against Rubisco activase (Fig. 4). This panel is representative of three independent experiments

Table 2 Effect of nano-anatase TiO_2 on the purified Rubisco activase activity of spinach

	Control	Bulk-TiO_2	Nano-anatase TiO_2
Rubisco activase activity (μ mg^{-1} protein)	0.51 ± 0.02[a]	0.55 ± 0.02[a]	1.27 ± 0.02[b]

The indexes listed above were determined by the assays described in 'Materials and methods'. The data presented are an average of the recordings from five independent experiments. a and b indicates that the differences of values followed by different letters in the same line are significant at $P = 0.05$ level (t test).

concentrations of Rubisco activase in the chloroplast stroma may possibly provide an environment conductive to self-association and cause the enhancement of its ability to function efficiently in vivo (Kim and Portis 2005). We therefore speculate that the nano-anatase TiO_2 treatment might also have an effect on the activity of Rubisco activase.

Activity of the purified Rubisco activase

For the purpose of proving our speculation, the activities of the purified Rubisco activase from spinach were measured, and the results are listed in Table 2. The activity of pure Rubisco activase in nano-anatase TiO_2-treated spinach is significantly higher than the control, by up to 2.5 times, whereas the activity of pure Rubisco activase with bulk TiO_2 treatment was only increased 9%, suggesting that nano-anatase TiO_2 treatment may cause higher concentrations of Rubisco activase, which provide an environment that lead to self-association and enhance the activity of pure Rubisco activase. As we know, the regulation of Rubisco activation in vivo is dependent on Rubisco activase, Therefore, with the Rubisco activase activity enhanced by nano-anatase TiO_2 in spinach, the Rubisco carboxylase activity would also be markedly improved, which was shown in Table 1.

Spectral analysis of purified Rubisco activase

The activity of pure Rubisco activase in nano-anatase TiO_2-treated spinach was significantly higher than that of control, we, thereby, suppose that it is related to not only the self-association of Rubisco activase, but also the structure of Rubiso activase. To prove this, spectroscopic measurements of purified Rubisco activase was carried out.

UV–Vis spectra of the purified Rubisco activase

UV–Vis spectra of the purified Rubisco activase are shown in Fig. 3. As we can see, the Rubisco activase purified from different treated spinach all have a characteristic peak of the tyrosine or tryptophan at 278 nm, and a characteristic peak of the amide bonds at 210–220 nm. However, the characteristic peak of the amide bonds from the nano-anatase TiO_2-treated spinach is red shifted by 5 nm compared with the control, and the characteristic peak of the amide bonds from the bulk TiO_2-treated spinach is nearly the same as control. The absorption intensity of the enzymes from the nano-anatase TiO_2- treated spinach is higher than that of the control, with the order of nano-anatase TiO_2 treatment > bulk TiO_2 treatment > control, suggesting that the microenvironment around tyrosine or tryptophan residue and the contents of the amide bonds of Rubisco activase are different from different treated spinach.

Fluorescence emission spectra of the purified Rubisco activase

Figure 4 shows that the fluorescence emission peak at 334 nm of the purified Rubisco activase from nano-anatase TiO_2, bulk TiO_2 treated spinach is not shifted; which is attributed to the tryptophan residues, indicating that the polar environment of fluorescent

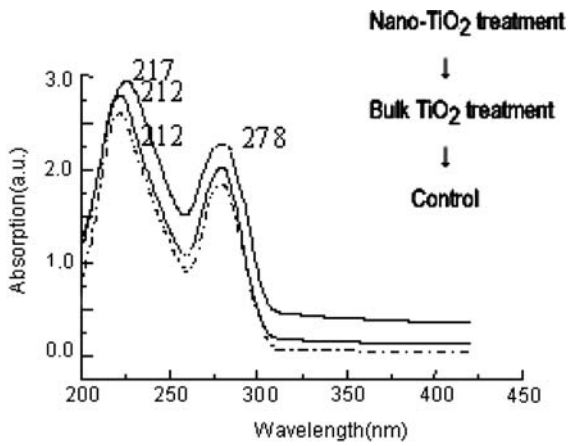

Fig. 3 Effects of nano-TiO_2 on UV–Vis spectra of the purified Rubisco activase from spinach

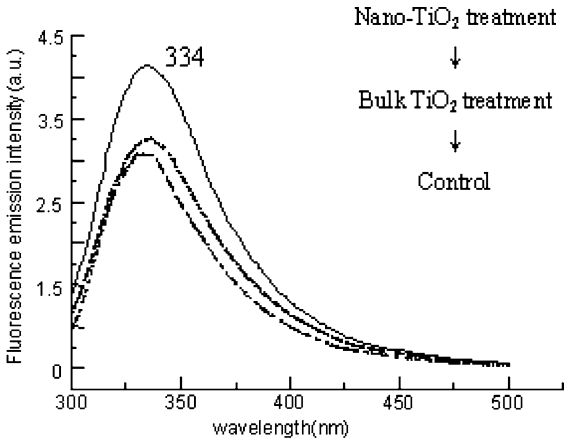

Fig. 4 Effects of nano-TiO_2 on fluorescence emission spectra of the purified Rubiso activase from spinach

aromatic groups does not change. But the intensity of the fluorescence emission peak of the purified Rubisco activase from nano-anatase TiO_2-treated spinach is 1.4 times that of control, which might be different from the microenvironment of Rubisco activase purified from different treated spinach.

Considering the enhancement of absorption and fluorescence emission of Rubiso activase by nano-anatase TiO_2 treatment, we believe that the nano-anatase TiO_2 treatment affected the microenvironment around tryptophan residue, which might lead to the change of the secondary structure of Rubiso activase.

Circular dichroism (CD) spectra of the purified Rubiso activase

To further confirm that nano-anatase TiO_2 treatment might affect the secondary structure of Rubiso activase, the CD spectra of the purified enzymes from spinach were detected and the results are shown in Fig. 5: the CD spectra of the purified Rubiso activase from nano-anatase TiO_2-treated, bulk TiO_2 treated spinach and the control have a nearly same negative peak at 216 nm, but the negative peak intensity of the purified Rubiso activase from the nano-anatase TiO_2-treated spinach is significantly higher than that of the control and bulk TiO_2 treated spinach. The results configured by using Perczel's method indicate that compared with the control, the α-helix, β-sheet and β-turn contents of Rubiso activase from nano-anatase TiO_2-treated spinach are

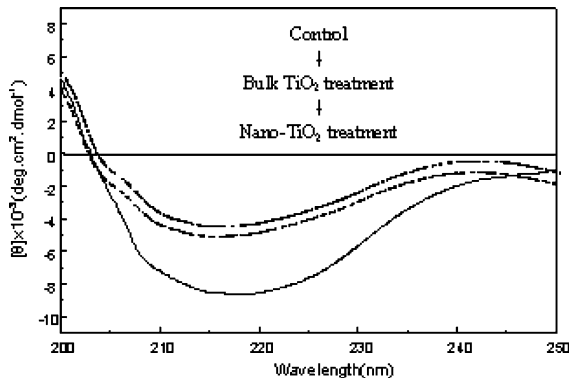

Fig. 5 Effects of nano-TiO_2 on ultraviolet CD spectra of Rubisco activase of spinach

increased by 12%, 18% and 13%, respectively, and the random coil contents are decreased by 57%. However, the secondary structure of Rubiso activase from bulk TiO_2 treated spinach is nearly the same as the control.

Conclusion

In the present research, the effects of nano-anatase TiO_2 on the activity and conformation of Rubiso activase were studied, and the results show that with the nano-anatase TiO_2 treatment, the amount of Rubiso activase is increased by 42%, and the activity of Rubiso activase is also obviously enhanced, by up to 2.5 times compared with the control, thereby, conducing to the great improvement of Rubsico carboxylation and the high rate of photosynthetic carbon reaction. Spectroscopic analysis indicates that the intensities of absorption and florescence of purified Rubiso activase from the nano-anatase TiO_2 treated spinach are significantly higher than that of the control, the secondary structure of the purified enzyme is also very different, the α-helix, β-sheet and β-turn contents are markedly increased, and the random coil contents are greatly decreased. Accordingly, it is suggested that the nano-anatase TiO_2 treatment could not only increase the amount of Rubiso activase which leads to self-association, but also improve the conformation of Rubiso activase, which resulted in the great enhancement of the enzyme activity and the rate of photosynthetic carbon reaction, improved spinach growth. However, bulk TiO_2 effect was not as significant as nano-anatase

TiO_2, as the grain size of nano-anatase TiO_2 (5 nm) is much smaller than that of bulk TiO_2, which entered spinach cell more easily.

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