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Effects of ozone on wild type and transgenic tobacco

J. GUO[1,2], X.F. LI[1], D.M. QI[1], S.Y. CHEN[1], Z.Q. LI[1]*, I. NIJS[3], Y.G. LI[1], G.S. LIU[1]*

Institute of Botany,Chinese Academy of Sciences, 20 Nanxincun, Beijing 100093, P.R. China[1]
Graduate Schoo1 of the Chinese Academy of Sciences, Beijing 100093, P.R. China[2]
Department of Biology, University of Antwerp, Campus Drie Eiken, Wilrijk B-2610, Belgium[3]

Abstract

Tocopherol cyclase (TC, encoded by gene *VTE1*) catalyzes the penultimate step of tocopherol synthesis. In this study we used wild type and transgenic tobacco plants overexpressing *VTE1* from *Arabidopsis* to examine the role of tocopherol in ozone sensitivity. Wild type plants responded to an 4-h exposure to 300 nmol mol^{-1} ozone by severe leaf necrosis while the transgenic lines exhibited limited injury. Compared with the wild type, *VTE1*-overexpressing plants had lower increase in hydrogen peroxide, malondialdehyde contents and ion leakage, and lower decrease of net photosynthetic rate 48 h following the ozone exposure. Transgenic plants also better maintained the structural integrity of the photosynthetic apparatus.

Additional key words: hydrogen peroxide, malondialdehyde, ion leakage, photosynthetic rate, tocopherol, ultrastructure.

Introduction

Ozone, as a strong oxidant, enters leaf mesophyll tissue by penetrating the stomata and rapidly induces formation of reactive oxygen species (ROS) in plants (Pellinen *et al*. 2002). This generates lipid peroxidation and membrane disruption, leading to modification of cell metabolism and decrease of photosynthetic capacity (Soldatini *et al*. 1998, Asada 2000), especially at higher concentrations. Plants use several strategies to combat ozone stress, including ozone avoidance by stomatal closure, and tolerance to ozone and/or its products. Ozone tolerance may result from activation of the detoxification system of ROS under ozone stress and/or from activation of repair processes. Plants have evolved elaborate enzymatic and nonenzymatic antioxidant systems to combat general oxidative stress (Conklin and Barth 2004, Foyer and Noctor 2005, Li *et al*. 2009). Vitamin E is involved in the latter type of defences (Dat *et al*. 2000, Alscher *et al*. 2002).

Tocopherols (α-, β-, γ-, and δ-tocopherol) are a group of ampliphilic lipid antioxidants, which collectively constitute vitamin E, and are synthesized exclusively in photosynthetic organisms (Wang and Quinn 2000). Many studies showed that α- and γ-tocopherols are considered more effective antioxidants than β and δ isoforms in *Arabidopsis* and lettuce (Shintani *et al*. 2002, Cheng *et al*. 2003, Della Penna 2005, Kumar *et al*. 2005, Chun *et al*. 2006, Lee *et al*. 2007). The antioxidant action of tocopherol depends on the breaking of the propagation of free-radical chains during lipid oxidation, which is crucial for scavenging ROS released during oxidative stress (Fryer 1992, Munné-Bosch and Alegre 2002, Hofius *et al*. 2004, Maeda *et al*. 2005, Della Penna and Pogson 2006). Moreover, the tocopherol content in plants correlates positively with tolerance to a variety of abiotic stresses (Munné-Bosch *et al*. 1999, Trebst *et al*. 2002, Collakova and Della Penna 2003, Rippert *et al*. 2004, Havaux *et al*. 2005, Kanwischer *et al*. 2005, Guo *et al*. 2006, Maeda *et al*. 2006, Abbasi *et al*. 2007, Bhargava *et al*. 2007, Collin *et al*. 2008, Liu *et al*. 2008).

Tocopherol cyclase (TC, encoded by gene *VTE1*) catalyzes the penultimate step of tocopherol synthesis and has been isolated from *Arabidopsis* and *Synechocystis* (Porfirova *et al*. 2002, Sattler *et al*. 2004, Della Penna and Pogson 2006). Vidi *et al.* (2006) have demonstrated that TC activity is restricted to the plastoglobules. Those are also the site of tocopherol accumulation in chloroplasts and take part in tocopherol synthesis.

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Abbreviations: c_i - intercellular CO_2 concentration; E - transpiration rate; g_s - stomatal conductance to water vapor; MDA - malondialdehyde; OTC - open-top chamber; PAR - photosynthetically active radiation; P_{Nmax} - light-saturated net photosynthetic rate; PPFD - photosynthetic photon flux density; ROS - reactive oxygen species; TC - tocopherol cyclase.
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* Corresponding authors; fax: (+86) 10 82590833; e-mail: lizq@ibcas.ac.cn and liugs@ibcas.ac.cn

Although the role of tocopherol in protecting against ozone toxicity is well known in animals (Wagner *et al.* 2007), there are no previous reports, to our knowledge, of how plants with modified levels of tocopherol respond to ozone. Metabolic engineering and nutritional genomics can be used in manipulating the metabolic flux and micronutrients in plants (Chen *et al*. 2006, Della Penna and Last 2006). In the current study, we use *VTE1* from *Arabidopsis* (Porfirova *et al*. 2002) overexpressing transformed tobacco plants to examine the hypothesis that elevated content of total tocopherol render tobacco plants tolerant to ozone dosages (300 nmol mol^{-1}) that normally induce injury. We analyse chloroplast ultrastructure, net photosynthetic rate, tocopherol, H_2O_2, malondialdehyde (MDA) and ion leakage.

Materials and methods

VTE1-overexpressing tobacco (*Nicotiana tabacum* L. cv. Wisconsin 38) lines TP27, TP12, and TP4 possessing higher, medium and lower content of tocopherol (Guo *et al.* 2006) and wild type plants were propagated through asexual buds. All plants were transplanted in *Vermiculite*, and grown in controlled conditions (16-h photoperiod, PPFD approximately 400 μmol m^{-2} s^{-1}, day/night air temperature of 25/20 °C and 50 - 70 % relative humidity). Plants were watered daily with Hoagland's nutrient solution. Eighth-leaf-stage plants were selected for uniformity and the third leaf from the top was used and labelled as the first fully expanded leaf.

The plants were placed in an open-top chamber (OTC) and exposed to 300 nmol mol^{-1} ozone for 4 h (10:00 - 14:00) under growth PPFD. The air dispensing system of the OTC was constructed according to Uprety (1998). Ozone was generated from air with an ozone generator (*JQ-6A*, *Telijie Co*., Beijing, China) and monitored with an ozone analyzer (*APOA-360*, *Horiba Ltd*, Kyoto, Japan). After fumigation, plants recovered 48 h. Control plants were kept in charcoal-filtered air during treatment. Plants were sampled for tocopherol content, H_2O_2 content, ion leakage, MDA content and net photosynthetic rate at experimental times 0 h (before ozone fumigation), 4 h (end of fumigation) and 48 h (after recovery in the OTC). Injury percentage and ultrastructure were determined only after 48 h. In all cases, the first fully expanded leaves of three different tobacco plants were chosen at every sampling time.

After 48-h recovery, the leaves from young (the third leaves from the top) to old (the seventh leaves from the top) wild type plants and transgenic plants were immediately excised and scanned (*Perfection 1240U*, *Epson*, Tokyo, Japan) into a computer. The area of visible necrotic damage on the leaf was calculated with image analysis software (*Image J*, National Institutes of Health, USA) (Nakajima *et al.* 2002) and expressed as percentage of total leaf area.

MDA, formed from the breakdown of polyunsaturated fatty acids, was determined by the thiobarbituric acid method of Hodges *et al.* (1999) with some modifications. The absorbance of supernatant was read at 532, 600 and 450 nm with the values of non-specific absorption at 600 and 450 nm being subtracted.

Membrane damage was assayed by measuring ion leakage from leaf discs (Rizhsky *et al.* 2002). Five leaf discs (10 mm diameter) were floated on 5 cm^3 of double distilled water at room temperature for at least 4 h. Following incubation, the conductivity of the bathing solution was measured. The tubes containing leaf discs were incubated with the bathing solution at 95 °C for 30 min. After cooling to room temperature the conductivity of the solution was re-measured. Ion leakage was expressed as the percentage of initial to final conductivity.

H_2O_2 was determined by the fluorescence procedure as reported by Chen and Gallie (2005). Leaf tissue (100 mg f.m.) ground in liquid nitrogen was extracted in 0.15 cm^3 of 25 mM HCl. H_2O_2 concentration was obtained by measuring relative fluorescence (excitation at 315 nm, emission at 425 nm) against a standard curve.

Tocopherols were extracted and quantified as described by Rippert *et al*. (2004), with detection and quantification according to standards (*Sigma-Aldrich*), and using fluorescence with excitation at 290 nm and emission at 325 nm.

Leaf gas change was measured on the green portion of leaves avoiding midveins, using an open infrared gas-exchange system (*GFS-3000*, *Heinz Walz*, Effeltrich, Germany). The leaf cuvette environment was controlled at photosynthetically active radiation (PAR) 1500 μmol m^{-2} s^{-1}, relative humidity approximately 60 %, and leaf temperature 25 °C. Transpiration rate (E), stomatal conductance to water vapor (g_s), light-saturated net photosynthetic rate (P_N) and intercellular CO_2 concentration (c_i) were calculated according to Von Caemmerer and Farquhar (1981).

Leaf tissue pieces (1 - 2 mm^2) were excised and immediately fixed in 3 % (m/v) glutaraldehyde in 50 mM sodium cacodylate buffer, pH 7.0, for 3 h. Samples were then washed three times for 30 min each in 50 mM sodium cacodylate buffer, pH 7.0, and were post-fixed in 1 % (m/v) OsO_4 overnight. At this stage, samples were dehydrated in increasing concentrations of acetone and then included in resin (*Epon*). A pre-inclusion at room temperature in increasing concentrations of resin dissolved in propylene oxide was followed by the final inclusion in freshly prepared resin followed by the polymerization at 45 °C for 3 h, and at 60 °C for 24 h. Ultra-thin (70 - 90 nm) sections were cut with an ultramicrotome (*Leica ULTRACUT R,* Solms, Germany) equipped with a glass blade. The ultrathin sections were mounted on uncoated copper grids (100 mesh) and contrasted by adding uranile acetate and an aqueous solution of lead nitrate before observation with

a transmission electron microscope (*JEM-1230*, Tokyo, Japan).

Statistical analysis was conducted using *SPSS-Win 13.0* (*SPSS Inc.*, Chicago, USA). For each sampling at 0, 4 or 48 h, the data were leaf injury, tocopherol content, MDA content, ion leakage, H_2O_2 content and photosynthetic parameters for wild type plants and transgenic plants (3 replicates in each case). One-way analysis of variance (*ANOVA*) was conducted to test for differences between the means of wild type plants and transgenic plants at the three times. The significance level was $\alpha = 0.05$ for all analyses.

Results

Under the experimental conditions used, the first visual symptoms were detectable as translucent spot-like lesions which developed into small necrotic intervenial areas after 48 h recovery in the young leaves of wild type and TP4 (about 6.07 %), but little in TP12 and TP27 (about 0.01 %). The percentage leaf injury rose to 23.2 % in old leaves of wide type and TP4, but only to 1.98 % in TP12 and TP27.

There was no significant difference in MDA content, ion leakage, and H_2O_2 content before ozone exposure. Ozone increased MDA content in all fumigated leaves but significantly less in TP27 and TP12 than in TP4 and wild type plants ($P < 0.005$). MDA content in both these types was restored to low levels similar to those before ozone treatment after 48 h recovery. These results indicate that the transgenic plants with overexpressing *VTE1* were able to increase the tolerance to ozone stress and decrease the level of membrane lipid peroxidation. The ozone exposure also increased ion leakage but likewise to a significantly smaller extent in TP27 and TP12. Wild type plants and TP4 thus apparently sustained the greatest membrane damage. Finally, ozone fumigation induced accumulation of H_2O_2 in leaves of all genotypes. Again the induction profile was different, with significantly lower contents reached at 48 h in the TP27 and TP12 characterized by higher tocopherol content. Both wild type plants and transgenic lines increased their total tocopherol content under ozone exposure, but TP27 and TP12 did so to a significantly greater extent and retained high tocopherol contents longer, which suggests that tocopherols protected tobacco plants from ozone stress.

The ozone fumigation significantly reduced net photosynthetic rate (P_N) in all genotypes, but in wild type and TP4 the inhibition developed more rapidly during the treatment than in TP27 and TP12. Transpiration rate (E), stomatal conductance (g_s) and P_N responded similarly to the ozone fumigation. Four hours of ozone exposure depressed the readings, but significantly more so in wild type and TP4. After 48 h, however, E, g_s and P_N were increased again, and these increases were significantly greater in TP27 and TP12. No significant differences were recorded in internal CO_2 concentration, c_i (Table 1).

There were no significant differences in chloroplast ultrastructure between wild type and TP27 before ozone treatment (data not shown). At 48 h, TP27 had significantly more chloroplasts per mesophyll cell, and greater chloroplast length and width than wild type (Fig. 1*a*,*b*), while cell size was little affected (Table 2). The number of starch grains per section, the area per starch grain, and the percentage of total starch grain area relative to chloroplast area per section were also significantly greater

Table 1. Physiological parameters in wild type and three transgenic tobacco lines (TP4, TP12 and TP27) before (0 h) and directly after (4 h) ozone exposure, and after recovery until 48 h. Means ± SE of three replicates.

Parameter	WT			TP4			TP12			TP27		
	0 h	4 h	48 h	0 h	4 h	48 h	0 h	4 h	48 h	0 h	4 h	48 h
MDA	29.42	53.71	31.40	31.63	52.16	33.49	30.23	45.73	30.59	29.22	37.26	29.62
[nmol g^{-1} DW]	±1.61	±2.87	±3.09	±1.72	±2.89	±3.10	±2.05	±2.33	±2.62	±2.25	±2.36	±2.27
Ion leakage	4.42	19.25	32.36	4.41	20.14	38.16	4.39	14.59	23.28	4.37	10.36	18.13
[%]	±0.53	±1.19	±3.01	±0.53	±1.22	±2.90	±0.54	±1.04	±1.93	±0.51	±0.97	±1.71
H_2O_2	3.94	27.35	54.23	3.87	25.12	49.37	3.54	17.26	43.19	3.54	15.14	33.02
[µmol g^{-1}(d.m.)]	±0.56	±2.06	±2.89	±0.55	±2.11	±2.05	±0.57	±2.03	±2.35	±0.60	±2.31	±2.30
Tocopherol	39.45	157.26	41.51	40.53	153.78	40.97	236.12	421.39	254.38	523.39	725.33	547.28
[µg g^{-1}(d.m.)]	±2.81	±13.16	±5.93	±2.92	±12.64	±5.87	±10.16	±27.89	±9.23	±22.79	±48.10	±41.93
E [mmol $m^{-2}s^{-1}$]	1.75	0.90	2.85	1.73	0.89	2.74	1.74	1.22	3.68	1.76	1.44	5.18
	±0.09	±0.07	±0.18	±0.09	±0.07	±0.16	±0.11	±0.10	±0.23	±0.18	±0.19	±0.31
g_s	93.03	39.29	158.27	91.16	37.62	159.54	93.59	42.52	231.43	90.79	68.34	312.56
[mmol $m^{-2}s^{-1}$]	±7.41	±5.99	±16.16	±8.14	±6.13	±13.26	±8.30	±5.28	±15.56	±10.44	±4.03	±14.53
P_N	7.15	4.46	8.83	7.24	4.26	8.24	7.93	5.13	11.59	8.49	6.46	16.77
[µmol $m^{-2}s^{-1}$]	±0.68	±0.11	±0.94	±0.73	±0.21	±0.82	±0.69	±0.16	±0.67	±0.78	±0.13	±0.78
c_i	254.82	236.39	288.59	246.73	227.48	278.93	228.54	265.53	286.05	220.85	271.76	289.52
[µmol mol^{-1}]	±8.59	±13.10	±11.55	±7.59	±14.10	±13.03	±8.13	±16.25	±17.84	±7.68	±18.08	±22.80

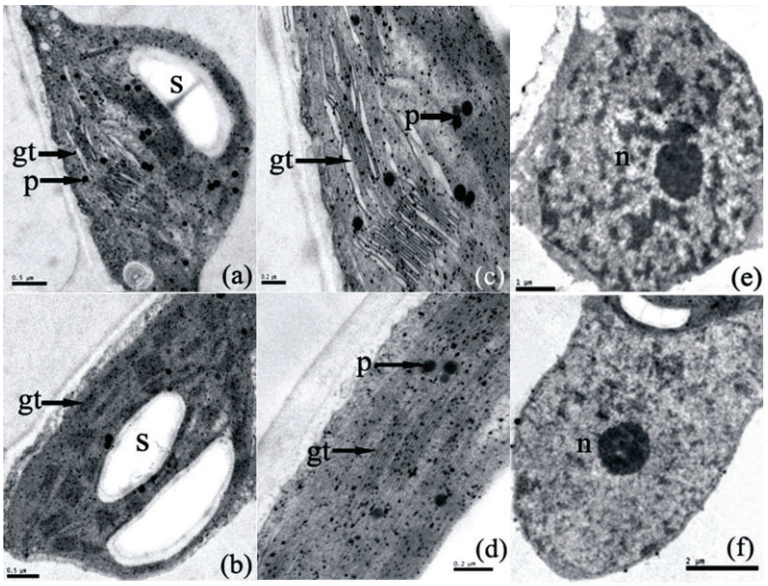

Fig. 1. Transmission electron micrographs sampled after recovery at 48 h, showing chloroplast and nuclear ultrastructure of leaves in wild type (*a*, *e*) and transgenic line TP27 (*b*, *f*) (*c* and *d* are enlargements from *a* and *b* respectively). s - starch, gt - grana in thylakoids, p - plastoglobuli, n - nucleus. *Bar* = 0.5 μm (*a*, *b*), 0.2 μm (*c*, *d*,), 1 μm (*e*) and 2 μm (*f*).

(Table 2). The number of plastoglobuli per section in TP27, on the other hand, was significantly lower than in WT. Apparent condensation of nuclear material into discrete patches distributed throughout the nucleus occurred in cells of ozone-treated wild type mature leaves, while the nuclei of TP27 remained intact (Fig. 1*e*,*f*).

Discussion

This study investigated whether tocopherols could reduce the effects of ozone on plants by comparing the responses of wild type and *VTE1*-overexpressing tobacco plants. To evaluate in detail the mechanisms underlying the ozone responses of plants with increased tocopherols and wild type plants, physiological parameters were analyzed relating to oxidative damage. Our results show that ion leakage and H_2O_2 content were substantially lower in transgenic plants with higher tocopherol level both upon and after exposure, indicating that lipids and membranes were better protected from oxidative damage (Table 1). MDA content was significantly higher in wild type plants during ozone treatment, and then declined to low levels again in all genotypes after 48 h recovery, reflecting the very short half-life of MDA in plants (Weber *et al*. 2004).

Table 2. Chloroplast ultrastructure of transgenic line TP27 and wild type exposed to 300 nmol mol^{-1} ozone during 4 h, and measured after recovery in O_3-free air for 48 h. Means ± SE of three plants (30 cells from leaves of each plant; * - differences significant at $P \leq 0.05$).

Chloroplast feature	WT	TP27
Number of chloroplasts [$cell^{-1}$]	10.86±0.74	16.00±1.46*
Chloroplast length [μm]	4.46±0.11	5.32±0.17*
Chloroplast width [μm]	2.19±0.05	3.05±0.10*
Number of starch grains [$section^{-1}$]	1.94±0.09	2.32±0.07*
Area per starch grain [μm^2]	2.96±0.25	8.09±1.77*
Starch grain/chloroplast area [%]	26.31±0.01	40.74±0.01*
Number of plastoglobuli [$section^{-1}$]	14.00±6.77	6.10±2.67*
Cell size [μm^2]	499.40±64.3	447.70±66.3

The increase of total tocopherol content during ozone treatment was proposed to be related to its function in scavenging ROS and preventing lipid peroxidation. We demonstrated that *VTE1*-overexpressing tobacco plants exhibited enhanced tolerance to ozone-induced oxidative stress, as evidenced by reduced leaf necrosis, reduced production of H_2O_2 (Table 1), and the conservation of chloroplast ultrastructure (Fig. 1). The enhanced tolerance in these lines can be ascribed not only to the higher content of tocopherol, but probably also to the longer duration of elevated level after exposure. Since there is substantial evidence that ozone reduces photosynthesis (Pell *et al*. 1994; Table 2), the plastid is likely to be a target of ozone related toxicity.

Kanwischer *et al*. (2005) have shown that *VTE1* overexpression in *Arabidopsis* could modify the levels of various antioxidants. Other authors pointed out that other mechanisms might compensate to fulfill the protective role when tocopherol is absent (Sattler *et al*. 2004, 2006, Havaux *et al*. 2005, Maeda *et al*. 2006). In cooperation with other antioxidants, tocopherol plays a part in reducing ROS levels in photosynthetic membranes, limiting the extent of lipid peroxidation and protecting membrane

lipids (especially polyunsaturated fatty acids) from oxidative damage by scavenging lipid peroxy radicals and by quenching or chemically reacting with ROS (Schneider 2005). Termination of polyunsaturated fatty acid free radical chain reactions by tocopherol occurs by donation of a hydrogen atom from the tocopherol ring hydroxyl resulting in a 'tocopherol radical'. In this way tocopherol could contribute to preservation of an adequate redox state in chloroplasts, and to maintaining intact thylakoid membrane structure and function during plant development, including periods of stress (Munné-Bosch and Alegre 2002, Sattler *et al*. 2004, Della Penna and Pogson 2006).

The visible injury that occurred in our tobacco plants under ozone exposure resembles the programmed cell death (PCD) associated with the hypersensitive response (HR) observed in plants after pathogen attack (Keen 1992, Pasqualini *et al*. 2003). In the HR, localized PCD is triggered by a burst of ROS (Keen 1992, Mehdy 1994). Huge accumulation of ROS was observed also in the current experiment on acute ozone, both during exposure and recovery after terminating fumigation, which further supports the suggestion of PCD in ozone stressed plants. Our results show that the visible injuries provoked by acute ozone fumigation were more severe in wild type than in transgenic plants. Ozone treatment induced aggregates of condensed chromatin in wild type, lending further support to the hypothesis of PCD under ozone fumigation, while chromatin had a normal morphology in ozone exposed transgenics (Fig. 1*e*,*f*). These data indicate that PCD might be promoted immediately at the end of the stress treatment in wild type. Contrastingly, transgenic plants with elevated total tocopherol content could scavenge more ROS in plant cells and as a result PCD might be boosted later and more slowly compare to the wild type. The gas exchange parameters demonstrated that elevated tocopherol content allowed transgenics to largely maintain intact photosynthetic apparatus and carbon fixation under elevated ozone. These results are supported by previous reports (Trebst *et al.* 2002, Bergmüller *et al*. 2003, Havaux *et al*. 2005, Munné-Bosch 2005, Abbasi *et al*. 2007).

Ultrastructural observations revealed that chloroplast membranes in wild type exposed to acute ozone were often disrupted. Moreover, thylakoidal systems shrank and part of the chloroplast remained virtually free of thylakoids. The thylakoid membranes also became incompact (Fig. 1*b*,*d*) and the number of thylakoids was reduced, which in turn explains the strong reduction in photosynthesis measured in mature leaves. In transgenic plants, on the other hand, the chloroplast envelope was still present, and the thylakoids were regularly appressed and stacked (Fig. 1). Protection of the photosynthetic capacity by tocopherol thus clearly arises at least partly from maintaining the structural integrity of the photosynthetic apparatus. The greater accumulation of starch in chloroplasts of transgenics may act as a mechanism for storing C, thereby expanding sink capacity.

In conclusion, our results show that *VTE1*-over-expression enhanced total tocopherol production, decreases necrosis, reduces injury to membranes, and avoids depression of photosynthesis in tobacco exposed to acute ozone stress. Based on our results, crop productivity in regions with high ozone concentrations could be safeguarded by enhancing tocopherol contents in plants to reduce harmful ozone effects. Better insight in ozone impact pathways should prove useful in plant breeding efforts and strategies to secure food supply in a future environment.

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