BIOLOGIA PLANTARUM 57 (2): 380-384, 2013 DOI: 10.1007/s10535-012-0285-x

BRIEF COMMUNICATION

Exogenous application of trehalose induced H_2O_2 production and stomatal closure in *Vicia faba*

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Abstract

Trehalose can reduce stomatal aperture by a hydrogen-peroxide-dependent pathway in *Vicia faba* L. (cv. Daqingpi) resulting in significantly lower values of net photosynthetic rate (P_N), stomatal conductance (g_s), and transpiration rate (E). At 8 and 24 h, the lower P_N in trehalose-treated plants was accompanied by significant decrease in intercellular CO_2 concentration (c_i) suggesting that the reduction of P_N was caused by stomatal limitation. At 48 and 72 h, trehalose decreased apparent carboxylation efficiency (P_N/c_i) and did not decrease c_i and g_s compared with controls; therefore the reduction in photosynthesis was caused by non-stomatal limitation. Trehalose treatment resulted in significantly higher effective photochemical efficiency of PS II (Φ_{PSII}) and did not affect maximum photochemical efficiency of PS II (F_v/F_m). At 24, 48, and 72 h, trehalose decreased non-photochemical quenching (NPQ) and increased photochemical quenching (qP). Our results suggest that trehalose did not damage photosynthetic reaction centers.

Additional key words: broad bean, chlorophyll fluorescence, gas exchange, net photosynthetic rate, stomatal and non-stomatal limitations, transpiration rate.

Drought stress can cause great damage to plant growth (Niu *et al*. 2004). One approach to improve the drought tolerance of plants is accumulation of osmoprotectant compounds such as glycine betaine, sorbitol, and trehalose (Almeida *et al.* 2007). However, so far few studies have explored the effects of trehalose on photosynthetic characteristics.

Trehalose (α-D-glucopyranosyl-[1,1]-α-D-glucopyranoside) is a nonreducing disaccharide widely distributed in nature (Avonce 2005, Iordachescu and Imai 2008), where it serves primarily as a protectant against environmental stresses (Wiemken 1990, Crowe *et al*. 1998, Wingler 2002). Multiple studies have revealed that transgenic plants overexpressing microbial trehalose biosynthesis genes have the ability to improve stress tolerance (Pilon-Smits *et al*. 1998, Garg *et al*. 2002, Iordachescu and Imai 2008, Li *et al*. 2011). Although the mechanism underlying the enhanced stress tolerance of these transgenic plants is still unknown, stomatal factors may play an important role. Trehalose is the starting point for chitin synthesis and chitin can reduce stomatal aperture by increasing reactive oxygen species (ROS) content in plants (Paul *et al.* 2008, Zhou *et al.* 2012). ROS, *e.g.* hydrogen peroxide plays a key role in regulating stomatal movements (Zhang *et al*. 2001). Although the role of H_2O_2 in abscisic acid (ABA) signaling in guard cells has been clearly examined in plants (Zhang *et al*. 2001), it is not known whether H_2O_2 acts as a second messenger in the regulation of stomatal function in response to trehalose.

Transgenic *Arabidopsis* plants that produce trehalose have different photosynthetic characteristics compared with wild-type plants (Almeida *et al*. 2007). The plants expressing *E. coli* trehalose phosphate phosphatase (TPP) have lower photosynthetic capacity than wild-type or plants expressing *E. coli* trehalose phosphate synthase (TPS), but they have larger biomass (Paul *et al*. 2008).

Received 8 January 2012, *accepted* 22 August 2012.

Abbreviations: ABA - abscisic acid; c_a - ambient CO_2 concentration; c_i - intercellular CO_2 concentration; E - transpiration rate; F_v/F_m - variable to maximum fluorescence ratio (maximum photochemical efficiency of photosystem II); g_s - stomatal conductance; NPQ - non-photochemical quenching; PAR - photosynthetically active radiation; P_N - net photosynthetic rate; P_N/c_i - apparent carboxylation efficiency; qP - photochemical quenching; ROS - reactive oxygen species; TPP - trehalose phosphate phosphatase; TPS - trehalose phosphate synthase; Φ_{PS2} - effective photochemical efficiency of PS II.

Acknowledgements: This work was financed by National High Technology Research and Development Program of China (2011AA100503). The first two authors contributed equally to this study.

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Pilon-Smits *et al*. (1998) and Almeida *et al*. (2007) evaluated chlorophyll fluorescence and found that, when subjected to drought or heat stress, transgenic plants exhibited a better photosynthetic performance. However, excessive accumulation of trehalose can cause multiple phenotypic and physiological alterations, such as growth defects, larger or lancet-shaped leaves, aberrant root development, and fewer seeds or sterility (Romero *et al*. 1997, Garg *et al*. 2002, Avonce *et al*. 2005, Stiller *et al*. 2008).

To solve the problem caused by excessive accumulation, we can use exogenous trehalose. Exogenous trehalose considerably improved drought or heat tolerance of plants by protecting thylakoid membranes, photosynthetic capacity, water relation attributes, and antioxidant defense mechanism (Luo *et al*. 2010). In this study, our objectives were *1*) to demonstrate whether trehalose may increase H_2O_2 production in guard cells inducing stomatal closure, and *2*) to examine whether the exogenous application of trehalose as a foliar spray may regulate the gas exchange attributes and chlorophyll fluorescence.

Vicia faba L. cv. Daqingpi was used for the present study and was grown in an open chamber of Zhejiang University (Zijingang Campus) at Hangzhou (120° 2′ E, 30° 3′ N), Zhejiang Province for 5 weeks. Seeds were soaked in water for 4 d and then sown in plastic pots (12 cm diameter × 14.5 cm height) filled with *Vermiculite*, *Perlite*, and soil (1:1:1 by volume) in April. The plants were irrigated daily and grown under natural irradiance.

Four leaf disks (5 mm in diameter) per treatment from the fully expanded young leaves were collected from 4 different plants. Epidermal peels were stripped carefully from the abaxial surface and immediately put into Mes/KCl buffer (10 mM Mes, 50 mM KCl, pH 6.15). These epidermal peels were incubated under irradiance of 300 μmol m^{-2} s^{-1} and temperature of 22 °C for 2 h to induce stomata opening, and were then transferred to different concentrations of trehalose (0, 10, 25, 50, and 100 mM) for further 1 h. We measured 50 stomatal apertures for each treatment on digital images captured using a *DSZ5000X* microscope (*UOP*, Chongqing, China) fitted with a *Canon PowerShot G10* camera.

Hydrogen peroxide production in guard cells was monitored using 2',7'-dichlorofluorescein diacetate (H_2DCF-DA) as previously described (Desikan *et al*. 2004b). The abaxial epidermal strips were treated as mentioned above. Then, they were loaded with H_2DCF-DA (50 μM) in Mes/KCl buffer for 15 - 20 min in darkness. Then the strips were washed with Mes/KCl buffer three times, and imaged with a *DSZ5000X* microscope. The fluorescence was quantified using *Image Pro Plus 6.0* software (*Media Cybernetics*, Silver Springs, MD, USA). Average fluorescence of control guard cells was set as 100 %. The fluorescence of treated cells was calculated as the ratio of treatment to control (Desikan *et al*. 2002).

The plants sprayed with 100 mM trehalose dissolved in water and the controls sprayed with water were used for chlorophyll fluorescence assay and gas exchange measurements. Eight leaves were randomly collected from four plants at 0 h (before trehalose spraying), 8, 24, 48, and 72 h after treatment. Two leaves were collected from one plant. The leaves used for chlorophyll fluorescence assay were detached from the plants during the experiment. Maximum photochemical efficiency of PS II (F_v/F_m), effective photo- chemical efficiency of PS II (Φ_{PSII}), non-photochemical quenching (NPQ), and photochemical quenching (qP) were analyzed with the *MAXI* version of the *IMAGING-PAM M*-series chlorophyll fluorescence system (*Heinz-Walz*, Effeltrich, Germany). Plants were dark adapted prior to measurements for 30 min. The details can be obtained from Kościelniak and Biesaga-Kościelniak (2006). Saturation pulses were given every 20 s (Da Silva *et al*. 2007).

Leaf gas exchange (net photosynthetic rate, P_N; transpiration rate, E; stomatal conductance, g_s; intercellular CO_2 concentration, c_i) in intact leaves were measured with a portable photosynthetic open-system (*CI-340*, *CID*, Camas, WA, USA) (Schlosser *et al.* 2012). Before measurements, the plants were sufficiently irradiated for more than 1 h. During measurements, the leaf chamber parameters were: irradiance 800 μmol m^{-2} s^{-1}, leaf temperature 25 °C, and ambient CO_2 concentration (c_a) 400 μmol mol^{-1}. Apparent carboxylation efficiency was calculated as P_N/c_i (Flexas *et al.* 2001).

Differences among treatments were compared using Tukey HSD (honestly significant difference) test. All the statistical analyses were performed using the *SPSS 10.0* package (*SPSS*, Chicago, USA).

Previous studies have proved that the movement of guard cells was clearly inhibited by trehalose (Dittrich and Mayer 1978). Here, we proved that trehalose induced stomatal closure in a dose-dependent manner (Table 1) and caused an elevation in the H_2O_2 content in guard cells (Table 1). H_2O_2 as a plant signaling molecule plays an important role in stomatal movements (Desikan *et al.* 2004a). H_2O_2 modulates signaling proteins, activates Ca^{2+}

Table 1. Effects of different concentrations of trehalose (0 - 100 mM) on stomatal aperture and H_2O_2 content (H_2DCF-DA fluorescence) in epidermal strips from *Vicia faba* plants. Means ± SE (n = 50), ** and * - significant difference at the $P < 0.01$ and $P < 0.05$ between the treated and control plants, respectively.

Parameters	0 mM	10 mM	25 mM	50 mM	100 mM
Stomatal aperture [μm]	11.20±0.90	8.08±0.78**	6.80±0.87**	4.63±0.66**	3.90±0.60**
H_2DCF-DA fluorescence [% control]	100.00±16.53	318.17±41.54*	566.32±62.24**	639.97±48.43**	1033.62±52.01**

Table 2. Effective photochemical efficiency of PS II (Φ_{PSII}), maximum photochemical efficiency of PS II (F_v/F_m), non-photochemical quenching (NPQ), photochemical quenching (qP), net photosynthetic rate (P_N) [μmol m^{-2} s^{-1}], stomatal conductance (g_s) [mmol m^{-2} s^{-1}], intercellular CO_2 concentration (c_i) [μmol mol^{-1}], transpiration rate (E) [mmol m^{-2} s^{-1}], and apparent carboxylation efficiency (P_N/c_i) of fully expanded control and trehalose (100 mM) treated leaves measured at 0 h (before trehalose spraying), 8, 24, 48, and 72 h after trehalose treatment. Means ± SE (n = 8). ** and * - significant difference at the $P < 0.01$ and $P < 0.05$ between the treated and control plants, respectively.

Parameters		0 h	8 h	24 h	48 h	72 h
Φ_{PSII}	control	0.60±0.004	0.57±0.003	0.57±0.003	0.58±0.003	0.56±0.003
	trehalose	0.60±0.004	0.59±0.005**	0.58±0.004	0.59±0.003**	0.58±0.004**
F_v/F_m	control	0.81±0.002	0.81±0.002	0.82±0.003	0.82±0.002	0.82±0.002
	trehalose	0.81±0.002	0.82±0.002	0.82±0.001	0.83±0.001	0.82±0.003
NPQ	control	0.63±0.007	0.75±0.007	0.76±0.005	0.76±0.004	0.87±0.007
	trehalose	0.68±0.010**	0.77±0.014	0.70±0.007**	0.73±0.004**	0.79±0.008**
qP	control	0.88±0.002	0.83±0.005	0.87±0.005	0.88±0.004	0.88±0.004
	trehalose	0.85±0.003**	0.86±0.003**	0.90±0.004**	0.91±0.004**	0.91±0.003**
P_N	control	8.86±0.100	8.58±0.800	9.27±0.184	7.88±0.232	7.01±0.188
	trehalose	9.20±0.129	6.03±0.253**	6.75±0.176**	6.75±0.190**	6.47±0.164*
g_s	control	196.80±5.217	181.42±8.959	136.22±6.757	104.68±4.844	79.04±3.291
	trehalose	187.29±3.948	99.57±9.244**	94.28±4.201**	104.93±3.230	74.39±3.994
c_i	control	266.94±2.673	258.52±2.565	261.49±2.387	252.94±5.442	217.74±3.760
	trehalose	270.91±1.727	230.70±6.382**	245.85±2.083**	261.06±3.142	230.19±3.349*
E	control	5.57±0.175	4.57±0.108	4.38±0.282	3.49±0.218	2.67±0.110
	trehalose	6.10±0.104	3.93±0.241**	3.06±0.170**	3.36±0.117	2.34±0.115
P_N/c_i	control	0.03±0.000	0.04±0.002	0.03±0.002	0.04±0.001	0.04±0.001
	trehalose	0.03±0.001	0.04±0.006	0.03±0.008	0.03±0.001**	0.03±0.002**

channels, inhibits K^+ channels, and induces cytosolic alkalinization in guard cells (Desikan *et al*. 2004a, Wang and Song 2008) which trigger water efflux and result in stomatal closure.

Chlorophyll fluorescence reflects the primary reactions of photosynthesis (Sayed 2003). Trehalose had no effect on F_v/F_m ratio in plants that indicated avoiding the impairment of the function of PS II. At 24, 48, and 72 h after treatment, a lower NPQ was observed in the treated leaves (Table 2) denoting a decrease in the energy dissipation through non-photochemical processes (Zlatev and Yordanov 2004). Higher Φ_{PSII} values in the trehalose treated plants than in the controls may reflect variation in the efficiency of carbon fixation (Table 2) suggesting that trehalose may increase the rate of CO_2 assimilation. However, our results showed that trehalose decreased P_N (Table 2). Increased Φ_{PSII} with lower P_N suggests that there are alternative electron sinks (Da Silva and Arrabaça 2004). There are two mechanisms that may be involved in lower P_N in this case: Mehler reaction or photorespiration. Trehalose caused stomatal closure and reduction in CO_2/O_2 ratio (Medrano *et al.* 2002). Under such conditions, O_2 acts as an alternate acceptor of electrons from the thylakoid electron transport chain, resulting in the formation of superoxide radical and other ROSs through the Mehler reaction (Zhang *et al.* 2001, Medrano *et al*. 2002). In our study, simultaneous decrease in P_N and increase in qP may also indicate that molecular oxygen acts as an efficient electron acceptor, reoxidizing the plastoquinone pool and maintaining high qP (Ribeiro *et al*. 2004). Thus, the Mehler reaction may play a major role in electron consumption in trehalose treated plants.

It is important to note that though trehalose may improve photosystem (PS) II function, it resulted in a significant reduction of P_N (Table 2). According to indications provided by g_s and c_i, we found that the lower P_N was due to stomatal closure at 8 h and 24 h after spraying trehalose (Table 2). Dittrich and Mayer (1978) reported that trehalose may cause stomatal closure. One potential explanation is that trehalose induced increase in H_2O_2 in leaf tissues (Table 1). Exposure of *V. faba* guard cells to exogenous H_2O_2 induces stomatal closure and activates Ca^{2+} influx currents (Desikan *et al.* 2004a, Yang *et al*. 2012). We proposed that similar responses occurred in broad bean plants after application of trehalose to the leaves which contributed to reduction in g_s, P_N, and E.

At 48 and 72 h after trehalose treatment, the lower P_N was not accompanied by decrease in c_i (Table 2) and higher Φ_{PSII} was observed (Table 2) suggesting that the reduction in photosynthesis was also caused by non-stomatal limitation. The mechanism of non-stomatal limitation is thought to include: *1*) lower efficiency of the photochemical reactions; *2*) lower carboxylation efficiency (Kanechi *et al.* 1996, Nunes *et al.* 2008). The excessive radiation energy can induce photoinhibition of PS II (Kanechi *et al*. 1996, Nunes *et al.* 2008). But in our studies, chlorophyll fluorescence parameters showed that PS II was not damaged (Table 2). On the other hand, trehalose significantly decreased P_N/c_i at 48 and 72 h (Table 2) which showed that trehalose probably decreased

RuBP regeneration and RuBP carboxylation efficiency (Flexas *et al*. 2001, Nunes *et al*. 2008). Decreases in the RuBP regeneration capacity may be a result of insufficient supplies of ATP (Kanechi *et al*. 1996, Nunes *et al*. 2008). When P_N is limited by ATP content, the excess of excitation energy could be dissipated *via* NPQ (Nunes *et al*. 2008) resulting in increases in NPQ. However, our study showed that NPQ was decreased (Table 2). To prevent photodamage, Mehler ascorbate peroxidase pathway may be involved (Nunes *et al*. 2008). Decreased photosynthetic rate may be result of increased Mehler reaction rate.

Taking together, we demonstrate that trehalose can reduce stomatal aperture by a H_2O_2-dependent pathway. The reduction of P_N in *V. faba* L. may be due to stomatal limitations after short-term trehalose treatment (8 and 24 h). The Mehler reaction may be the major non-stomatal limiting factor after longer treatment (48 and 72 h).

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