

Overexpression of zeaxanthin epoxidase gene from *Medicago sativa* enhances the tolerance to low light in transgenic tobacco

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Zeaxanthin epoxidase (ZEP) plays an important role in xanthophyll cycle which is a process closely related to photosynthesis. However, an impact of ZEP on low-light stress has not been studied. In this study, the functions of an alfalfa (*Medicago sativa*) zeaxanthin epoxidase gene, *MsZEP*, in response to low-light stress were investigated by heterologous expression in tobacco (*Nicotiana tabacum*). Under normal light conditions, the measured parameters were not significantly different between transgenic and wild-type (WT) plants except for non-photochemical quenching value and chlorophyll a content. However, the differences were detected under low-light stress. We found that *MsZEP*-overexpression tobacco grew faster than WT ($p \leq 0.05$). The leaf fresh weight and leaf area of transgenic plants were significantly higher, and the number of stomata was greater in *MsZEP*-overexpression tobacco. As for photosynthetic characteristics, quantum yield of PSII (Φ_{PSII}) and maximal photochemical efficiency of PSII (F_v/F_m) were not significantly different, whereas non-photochemical quenching (NPQ), net photosynthetic rate (Pn), stomatal conductance (Gs) and transpiration rate (Tr) of *MsZEP*-overexpression tobacco were significantly higher than in WT plants. However, no significant difference was detected between the two types of tobacco in chlorophyll and carotenoids content. In conclusion, *MsZEP* can improve the ability of tobacco to withstand low-light stress, which might be due to its stronger photosynthetic activity and the improvement of stomatal density under low light.

Key words: *Medicago sativa*, low-light tolerance, zeaxanthin epoxidase, overexpression

Received: 10 January, 2018; revised: 21 May, 2018; accepted: 15 July, 2018; available on-line: 08 September, 2018

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Abbreviations: Ax, antheraxanthin; Chl, chlorophyll; F_v/F_m , maximal photochemical efficiency of PSII; Gs, stomatal conductance; *MsZEP*, *Medicago sativa* zeaxanthin epoxidase gene; NPQ, non-photochemical quenching; Pn, net photosynthetic rate; WT, wild-type plants; Φ_{PSII} , quantum yield of PSII; ZEP, Zeaxanthin epoxidase; Zx, zeaxanthin; Vx, violaxanthin

INTRODUCTION

Light is the most important environmental factor which can influence the growth and development of plants (Zoratti *et al.*, 2014). The photosynthetic apparatus absorbs radiant energy and converts it to stable chemical energy which plants can use to grow and develop. Light intensity and quality directly affect photo-

synthesis of plants (Romanowska *et al.*, 2008; Lee *et al.*, 2007; Farquhar *et al.*, 2001; Sæbø *et al.*, 1995). Plants have evolved some mechanisms to adapt to different irradiance levels, which makes them capture and use the limited light efficiently while avoiding the potentially damaging effects of excessive irradiance (Kang & Zhu, 2003). The plants response to irradiance change includes changes in the number of chloroplasts per unit leaf area, altered leaf thickness and photosynthetic capacity per unit leaf area, leaf movement, altered xanthophyll cycle, chlorophyll (Chl) concentration and others (Pastenes *et al.*, 2005; Latowski *et al.*, 2004; Murchie & Horton, 1997).

In both higher plants and green algae, the xanthophyll cycle is involved in thermal energy dissipation, the main way of removal of the excess light energy (Hieber *et al.*, 2000; Bugos *et al.*, 1998; Demmig-Adams & Adams, 1996; Yamamoto & Higashi, 1978). In this cycle, there are three pigments to interconvert, including zeaxanthin (Zx), antheraxanthin (Ax) and violaxanthin (Vx) and two enzymes, zeaxanthin epoxidase (ZEP) and violaxanthin de-epoxidase which catalyze the cycle. Plants can dissipate excess light energy through these three components conversion. Zeaxanthin epoxidase (ZEP) is an important enzyme which catalyzes the conversion of Zx to Ax and Vx. This reaction is performed mainly under low light, while it weakens and even vanishes under strong light (Schwarz *et al.*, 2014; Wang *et al.*, 2008; Frommolt *et al.*, 2001). The conversion is closely related not only to xanthophyll cycle but also to ABA biosynthesis and carotenoid biosynthesis (DellaPenna & Pogson, 2006; Nambara & Marion-Poll, 2005). ZEP gene was studied in various plants including *Arabidopsis* (Barrero *et al.*, 2005; Xiong *et al.*, 2002), *Nicotiana plumbaginifolia* (Audran *et al.*, 1998; Marin *et al.*, 1996), tomato (Wang *et al.*, 2008) and rice (Agrawal *et al.*, 2001), but these studies mainly focused on the role of ZEP in ABA biosynthesis, during osmotic or strong-light stress and during seeds development. However, little is known about its response under low light.

Alfalfa is a major legume forage in the world because of its high nutritional value, high yield and wild adaptability. We previously isolated a ZEP gene from alfalfa and studied its role in transgenic tobacco under drought and salt stress (Zhang *et al.*, 2016). Xanthophyll cycle is closely related to photosynthesis and photoinhibition under low light and ZEP is involved in this cycle. Therefore, we further studied the physiological function of this gene in response to low-light stress using *MsZEP*-overexpression *Nicotiana tabacum* plants.

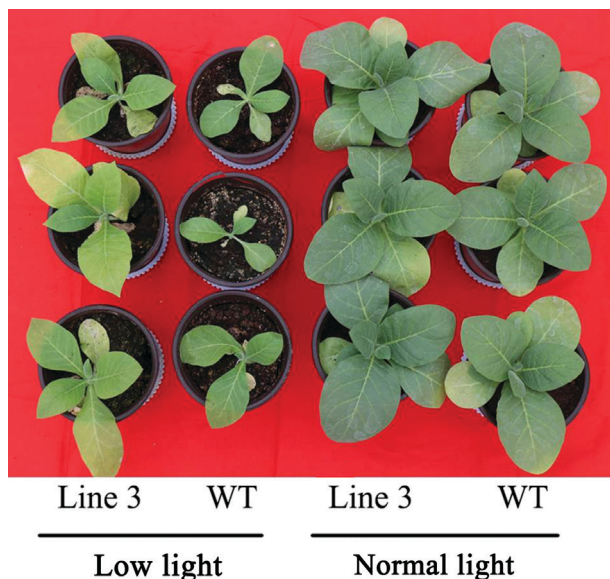


Figure 1. Effect of weak-light stress on the growth of WT and transgenic plants.

MATERIALS AND METHODS

Plant material and treatments. Wild-type (WT) tobacco (*Nicotiana tabacum*) and the *MsZEP*-overexpression line (Line 3) were previously described (Zhang *et al.*, 2016). Seeds of these two lines were surface-sterilized with 75% ethanol for 30 s, and 0.5% sodium hypochlorite solution for 10 min and then rinsed with sterile distilled water 4–5 times. Seeds of *MsZEP*-overexpression tobacco were germinated and grew on Murashige and Skoog medium (Murashige & Skoog, 1962) with 0.35% plant phytigel and 30 mg/L hygromycin B at 24°C under 16-h photoperiod for two weeks, and seeds of WT grew in the same conditions but without hygromycin B. These 14-day-old seedlings were transplanted to plastic pots filled with sandy soil and divided into two groups at random. One group was grown at 25°C and 40 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for 3 weeks before the transfer to 25°C and 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$. The other group was grown at 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$. After growing in soil for 6 weeks, plants were harvested and immediately frozen in liquid nitrogen and stored at -80°C until use.

Growth measurements. The leaves of WT and *MsZEP*-overexpression tobacco were harvested after two different treatments. Leaf fresh weight and area for each tobacco plant group were measured with electronic scales and a leaf-area meter, respectively. The density of stomata was calculated using an optical microscope.

Photosynthetic capability measurement. Chlorophyll fluorescence was measured using a pulse-amplitude-modulation chlorophyll fluorometer (Heinz-Walz-GmbH, Effeltrich, Germany). ΦPSII , F_v/F_m ratio and NP were recorded during a saturating photon pulse (4000 $\mu\text{mol m}^{-2} \text{s}^{-1}$) using a whole plant (Yang *et al.*, 2014). Gs, Tr and Pn of transgenic and WT plants were determined using a Li-COR 6400 system (Lincoln, NE, US) (Elhaddad *et al.*, 2014). Measurements were taken from three leaves harvested from three separate plants of each line.

Pigments measurements. Leaf pigments concentrations (Chl a, Chl b, carotenoids) were determined spectrophotometrically according to Arnon (1949) by homogenizing tissue in 80% acetone. Extract absorbance

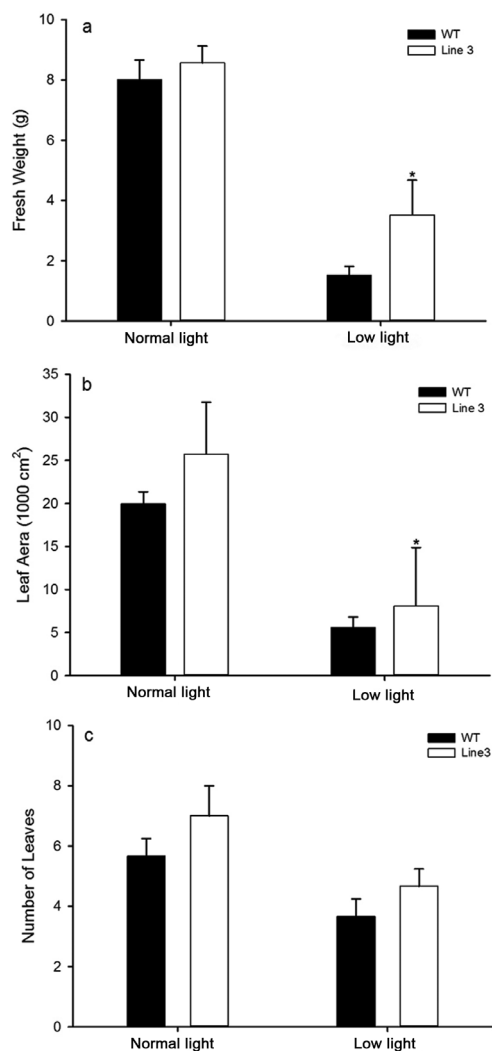


Figure 2. Effect of weak-light stress on leaf characteristics of WT and transgenic plants.

(a) Fresh matter of total leaves. (b) Total leaf size. (c) Leaves number. Bars represent the means \pm S.E. ($n=3$); asterisk indicates $p \leq 0.05$.

was measured at the wavelength of 645, 663 and 470 nm with 5100UV spectrophotometer (Shanghai, China).

Statistical analysis. All data are presented as the means \pm standard errors (S.E.) from three biological replicates of each experiment. Statistical significance was calculated using Student's *t*-test. The significant differences among various treatment groups are represented

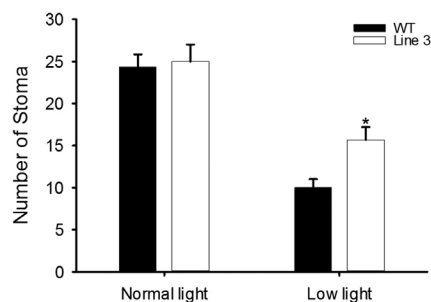


Figure 3. Effect of weak-light stress on the density of stomata of WT and transgenic plants.

Bars represent the means \pm S.E. ($n=3$); asterisk indicates $p \leq 0.05$.

by * for $p \leq 0.05$ and ** for $p \leq 0.01$. Analyses were performed with IBM SPSS Statistics 18.0 software. Figures were created using SigmaPlot 10.0 (Systat Software, Inc., Germany).

RESULTS AND DISCUSSION

Plant growth and development under low-light conditions

Light intensity plays an important role in plants growth and development (Pan & Guo, 2016). Under the maximum light intensity required by plants, the plant productivity decreased by 1% when the light intensity dropped by 1% (Wilson *et al.*, 1992). We observed that low light decreased the growth rate of tobacco, whereas overexpressing *MsZEP* enhanced the ability of tobacco to withstand the low-light stress (Fig. 1).

To further understand the effect of weak-light stress on the growth of both tobacco types, we determined the leaf fresh weight, the number of leaves, and leaf area. We found that the leaf fresh weight (Fig. 2a) and leaf area

(Fig. 2b) of both *MsZEP*-overexpression and WT tobacco decreased dramatically after weak-light treatment, but the leaf weight and leaf area of *MsZEP*-overexpression tobacco were significantly higher than in WT lines. The number of leaves of transgenic plants was higher than in wild-type (Fig. 2c). Some research reported that leaves in low light are larger than leaves in high light (Lichtenthaler *et al.*, 1981; Bjorkman *et al.*, 1972).

The number of stomata

Lichtenthaler and coworkers (Lichtenthaler *et al.*, 1981) reported that light intensity could change stomatal density. To understand the effect of *MsZEP* gene on the numbers of leaf cells and stomata, the lower epidermis of leaf was observed using an optical microscope. *MsZEP*-overexpression tobacco showed higher stomatal density under low light conditions, while the stomatal density was comparable between wild-type and *MsZEP*-overexpression plants under normal growth conditions (Fig. 3). ZEP catalyzes the conversion of Zx into Vx, which is an essential part of ABA biosynthesis (Nambara & Marion-Poll, 2005). As reported previous-

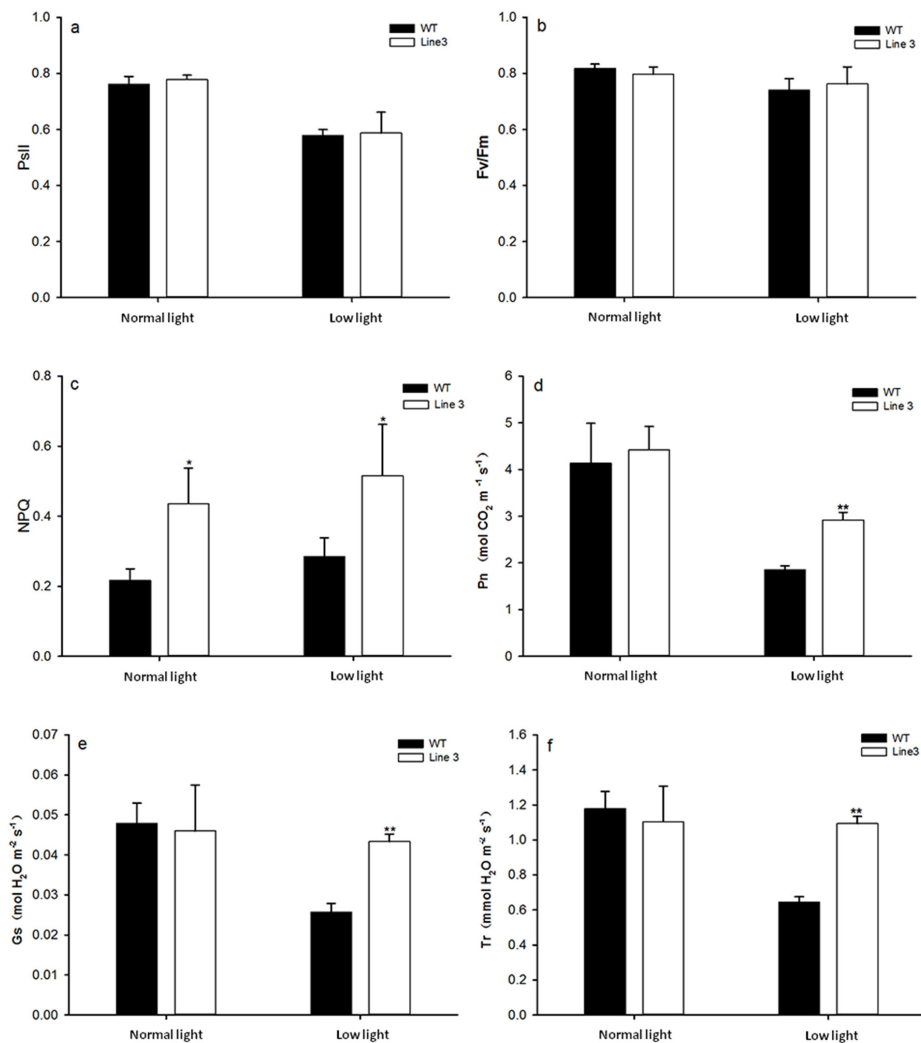


Figure 4. Effect of weak-light stress on the photosynthesis of WT and transgenic plants.

(a) Quantum yield of PSII. (b) Maximal photochemical efficiency of PSII. (c) Non-photochemical quenching. (d) Net photosynthetic rate. (e) Stomatal conductance. (f) Transpiration rate. Bars represent the means \pm S.E. (n=3); asterisk and double asterisk indicate $p \leq 0.05$ and $p \leq 0.01$, respectively.

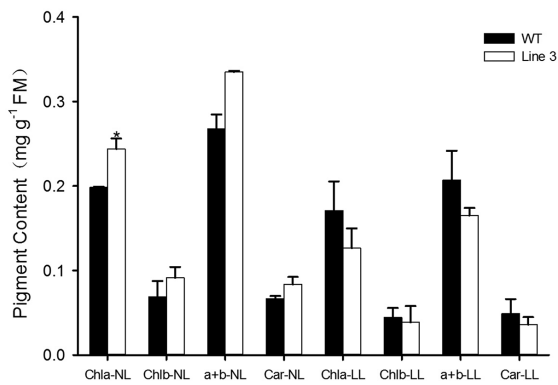


Figure 5. Effect of weak-light stress on pigment content of WT and transgenic plants.

Bars represent the means \pm S.E. ($n=3$); asterisk indicates $p \leq 0.05$; NL represents normal light, and LL represents low light.

ly, ABA content in *MsZEP*-overexpression tobacco was higher than in WT (Zhang *et al.*, 2016). The increased ABA content in *MsZEP*-overexpressors may increase stomatal density under low-light stress, while the phenomenon was not significant under normal growth conditions.

Photosynthetic characteristics

To investigate the effect of *MsZEP* gene on photosynthesis levels after weak-light treatment, photosynthetic activity of wild-type and transgenic tobacco was assessed. As shown in Fig. 4, the maximum quantum efficiency of PSII (F_v/F_m) value and maximum yield of PSII (Φ_{PSII}) value of the two plant types decreased after weak-light treatment, indicating a decrease in the rate of light energy conversion (Fig. 4a and 4b). This contributes to the decline of photosynthetic ability. However, the difference in Φ_{PSII} and F_v/F_m between WT and *MsZEP*-overexpression tobacco was not significant, neither under normal nor low light (Fig. 4a and 4b).

Xanthophyll cycle is tightly related to the dissipation of excess light in plants, and NPQ represents the energy which cannot be utilized in photosynthesis and is dissipated harmlessly as heat energy (Veres *et al.*, 2006; Müller *et al.*, 2001; Vasil'ev *et al.*, 1998). In our research, when plants were grown in normal-light condition after the low-light treatment, NPQ increased in both wild and *MsZEP*-overexpression tobacco, reaching 18.22% and 30.65%, respectively (Fig. 4c). In addition, the NPQ of *MsZEP*-overexpression tobacco was always higher than in wild-type under both conditions ($p \leq 0.05$) (Fig. 4c). Xanthophyll-cycle pigments (Vx, Ax, Zx) are involved in the process of NPQ of chlorophyll fluorescence which is the most important mechanism of photoprotection (Demmig & Adams, 1991). The increase of NPQ value may be due to the expression of *MsZEP* in transgenic plants. This indicates that overexpressing *MsZEP* may improve plants' sensitivity to light. Similar results were observed for zeaxanthin epoxidase gene in tomato (Wang *et al.*, 2008).

Under normal-light conditions, leaf net photosynthesis (Pn), stomatal conductance (Gs) and transpiration rate (Tr) displayed no significant difference between *MsZEP*-overexpression and WT tobacco (Fig. 4d, 4e and 4f). When plants were exposed to low light level, Pn decreased significantly, but in transgenic plants was higher than in WT ($p \leq 0.01$) (Fig. 4d), which suggests that overexpressing *MsZEP* enhances the photosynthetic ability

under weak light. The changes of Gs and Tr were similar to Pn (Fig. 4e and 4f). The higher stomatal density of *MsZEP*-overexpression tobacco may lead to the higher Gs. In addition, the higher Gs can contribute to the higher Pn and Tr. Therefore, the higher stomatal density may benefit photosynthesis and growth of plants.

Pigment content

Chlorophyll absorbs light energy during photosynthesis and the content of Chl a is three times higher than Chl b. Bell reported that the content of chlorophyll decreased under low-light stress applied for a long time (Bell & Danneberger, 1999), while Lakshmi and Singh revealed that the content of both Chl a and Chl b increased and the rate of Chl b increase was higher (Praba *et al.*, 2004; Singh *et al.*, 1988). In the present study, the concentration of Chl a, Chl b and carotenoids in wild-type and *MsZEP*-overexpression tobacco decreased after low-light stress (Fig. 5). However, the difference between *MsZEP*-overexpression and WT tobacco was not significant except for chlorophyll a content under normal-light condition. Therefore, *MsZEP* may enhance the tolerance of tobacco to weak light *via* other mechanism than by altering chlorophyll and carotenoids levels.

In conclusion, overexpressing *MsZEP* can improve the ability of tobacco to withstand low-light stress, because *MsZEP*-overexpression tobacco grew faster than WT. Although NPQ of *MsZEP*-overexpression tobacco was higher, the photosynthesis rate of *MsZEP*-overexpression tobacco was also higher than WT tobacco. This may be due to the higher density of stomata in *MsZEP*-overexpression tobacco.

Acknowledgements of Financial Support

This work was supported by the Project of National Natural Science Foundation of China (31572456; 31772660) and China Agriculture Research System (CARS-34).

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