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Overexpression of the alfalfa zeaxanthin epoxidase gene delays seed germination in transgenic tobacco

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Abstract

Zeaxanthin epoxidase (ZEP) plays important roles in plant growth and development due to its functions in abscisic acid (ABA) biosynthesis and in the xanthophyll cycle. Many studies have been exploring the roles of ZEP in seed germination and response to various environmental stresses. In this study, we reported that heterologously overexpressing the *ZEP* gene from *Medicago sativa* (*MsZEP*) in tobacco increased ABA content in tobacco mature seeds and negatively regulated seed germination. Enhanced ABA synthesis in seed embryo and delayed germination might also be related to the increased 9-*cis*-epoxycarotenoid dioxygenase (*NCED6*) expression. Moreover, we found that overexpression of *MsZEP* resulted in an increased expression of the *SOMNUS* gene but a decreased expression of the *DNA ligase 6* gene (*Lig6*) suggesting that *MsZEP* might affect seed proteome and DNA integrity. Furthermore, enhanced chlorophyll content in transgenic tobacco seedlings overexpressing *MsZEP* might be due to its function in the xanthophyll cycle and ABA biosynthesis.

Additional key words: abscisic acid, chlorophyll, *Medicago sativa*, *Lig6*, *NCED6*, *Nicotiana tabacum*, *SOMNUS*, xanthophyll cycle.

Introduction

Zeaxanthin epoxidase (ZEP) converts zeaxanthin to violaxanthin *via* the intermediate antheraxanthin and plays important roles in plant growth and development due to its functions in abscisic acid (ABA) biosynthesis and xanthophyll cycle (Nambara and Marion-Poll 2005). The vast majority of recent research attention has been dedicated to exploring the roles of ZEP in response to various environmental stresses in different plant species, including *Arabidopsis* (Barrero *et al.* 2005), *Nicotiana glauca* (Xiong and Zhu 2003), *Oryza sativa* (Agrawal *et al.* 2001), and *Medicago sativa* (Zhang *et al.* 2016).

The *ZEP* transcription is different among species and tissues under various environmental stresses. For example, the amount of *ZEP* transcripts increases in roots of tobacco and tomato during drought stress, whereas showing little changes in leaves (Thompson *et al.* 2000a, Agrawal *et al.* 2001). In alfalfa, *MsZEP* expression decreases in shoots

under drought, cold, heat, and ABA treatments, whereas the expression in roots increases first and then decreases under drought, heat, and ABA treatments (Zhang *et al.* 2016). The functions of ZEP in plant abiotic stress tolerance have been studied by using transgenic technology in many plants. It was reported that the *ZEP* gene is involved in the regulation of ABA biosynthesis in roots and contributes to plant response to drought (Audran *et al.* 2001). Overexpression of the *ZEP* gene increases sensitivity to high irradiance and chilling stress in tomato (Wang *et al.* 2008) and enhances salt and drought tolerance in *Arabidopsis* (Park *et al.* 2008).

Because of its high vulnerability to biotic and abiotic stresses, germination is the most critical phase in the plant life cycle (Rajjou *et al.* 2012). Abscisic acid is an important controlling factor during seed germination. It is known that ABA promotes seed dormancy and represses seed germination (Holdsworth *et al.* 2008, Je *et al.* 2014). The ZEP is the first enzyme that was identified as an ABA biosynthetic enzyme (Marin *et al.* 1996). Moreover, ABA-

Submitted 15 August 2018, last revision 24 February 2019, accepted 28 February 2019.

Abbreviations: ABA - abscisic acid; *Lig6* - DNA ligase 6; *MsZEP*: - *Medicago sativa* zeaxanthin epoxidase gene; *NCED* - 9-*cis*-epoxycarotenoid dioxygenase; *SOM* - *SOMNUS*; *WT* - wild-type; *ZEP* - zeaxanthin epoxidase.

Acknowledgments: This work was supported by the Project of National Natural Science Foundation of China (31272490; 31572456; 31772660), the China Agriculture Research System (CARS-34), and the Project of National Natural Science Foundation of Inner Mongolia (2017BS0305). Yuman Cao and Zhiqiang Zhang contributed equally to this work.

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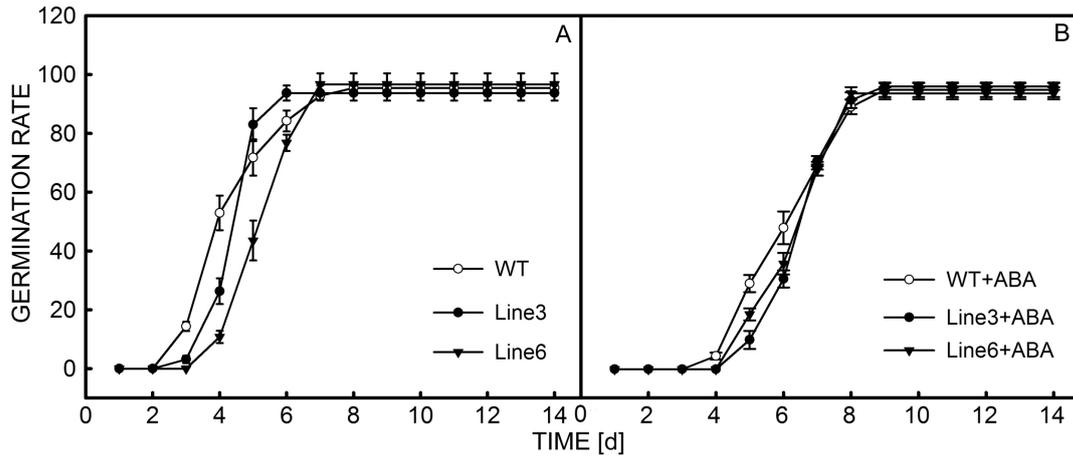


Fig. 1 The effect of *MsZEP* overexpression on seed germination. Germination rate of wild type (WT) and two *MsZEP*-overexpressing lines under normal growth conditions (A) and after treatment with 5 μM ABA (B). Line 3 and Line 6 - two *MsZEP*-overexpressing plant lines of T₂ generation. Means ± SEs, n = 3 sets of seeds, each containing 50 seeds.

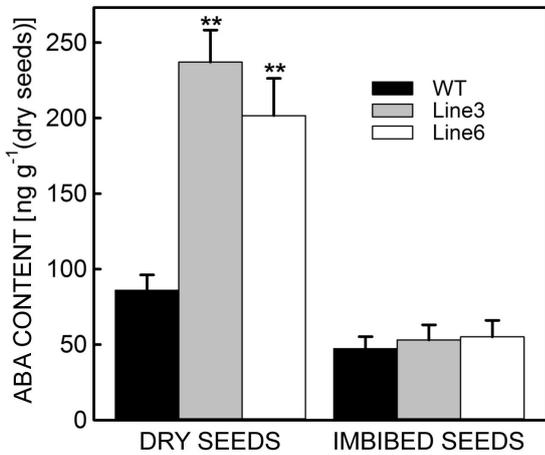


Fig. 2 The effect of *MsZEP* overexpression on abscisic acid (ABA) content in tobacco seeds. WT - wild-type tobacco; Line 3 and Line 6 - two *MsZEP*-overexpressing plant lines of T₂ generation. Means ± SEs, n = 3, * and ** - differences significant at $P \leq 0.05$ and 0.01 , respectively.

deficient mutants, such as *Arabidopsis thaliana aba1* and *Nicotiana plumbaginifolia aba2*, are defective in zeaxanthin epoxidation (Rock and Zeevaart 1991). Many studies have reported that plants overexpressing a *ZEP* gene exhibit an increased ABA content in different tissues (Audran *et al.* 2001, Park *et al.* 2008, Zhang *et al.* 2016). The role of *ZEP* on germination have been previously analyzed in mutants (Marin *et al.* 1996) and transgenic plants (Frey *et al.* 1999). It was demonstrated that this gene affects ABA content in seeds and then acts as a negative regulator of germination. An *MsZEP* expression is clearly tissue-specific and is highly expressed in green tissues in alfalfa (Zhang *et al.* 2016). In addition, *MsZEP* expression is induced by nodulation and abiotic stress treatment. Heterologous expression of *MsZEP* shows an enhanced ABA content and increased tolerance to drought and salt stresses in tobacco (Zhang *et al.* 2016). However, little is known about the effect of *MsZEP* on seed germination. In this study, we validated the function of *MsZEP* during seed germination by heterologous expression of *MsZEP* in tobacco.

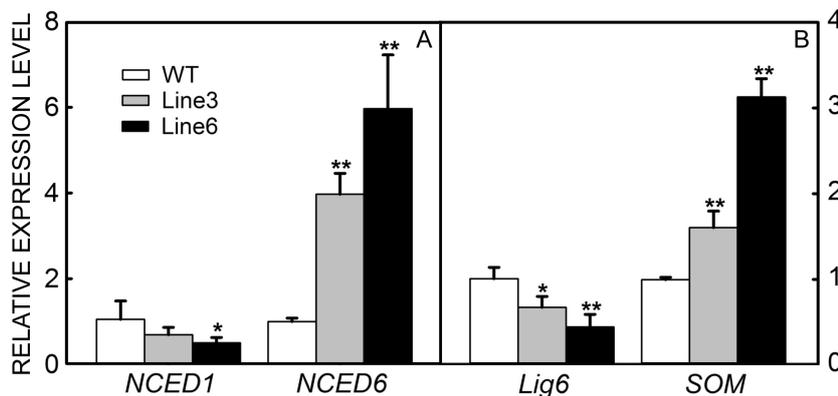


Fig. 3 The effect of *MsZEP* overexpression on the expression of genes involved in abscisic acid (ABA) biosynthesis and seed germination. A - The expression of genes involved in ABA biosynthesis in wild type (WT) and *MsZEP*-overexpressing seeds. B - The expression of genes involved in seed germination in WT and *MsZEP*-overexpressing seeds. Line 3 and Line6 - two T₂ *MsZEP*-overexpressing plant lines. Means ± SEs, n = 3, * and ** - differences significant at $P \leq 0.05$ and 0.01 , respectively.

Materials and methods

Plants and cultivation: Tobacco (*Nicotiana tabacum* L.) wild type (WT) and the lines overexpressing *MsZEP* controlled by the 35S promoter have been previously described (Zhang *et al.* 2016). To obtain transgenic seeds from these lines, T₁ tobacco seeds were firstly sown and germinated on a Murashige and Skoog medium containing 30 mg dm⁻³ hygromycin B at a temperature of 24 °C, an irradiance of 60 μmol m⁻² s⁻¹, and a 16-h photoperiod. Subsequently, 10-d-old plantlets were transferred to pots filled with sandy soil in a greenhouse with natural irradiance, an average temperature of 24 °C, and a relative humidity of 65 ± 5 %, and grown till maturity.

Germination assay: Seeds of wild type and T₂ transgenic lines were harvested on the same day from tobacco and dried for two weeks. Dried seeds were surfaced sterilized with 70 % (v/v) ethanol, rinsed with water, and air-dried. Seeds were imbibed on wetted filter paper supplemented with 0 (control) and 5 μM ABA in Petri dishes and then germinated at 24 °C and a 12-h photoperiod. Experiments were performed in triplicate, with 50 seeds counted for

each treatment. The number of germinated seeds in each Petri dish was counted every day for 14 days. Seeds with visible radicles were considered as germinated (Rajjou *et al.* 2012).

Quantification of ABA: Extraction and purification of ABA were carried out according to Park *et al.* (2008) and Zhang *et al.* (2016) with some modifications. In brief, about 50 mg of dry tobacco seeds or seeds imbibed in water for 8 h were frozen in liquid nitrogen and grounded to powder with a tissue lyser (*Qiagen*, USA), and then the samples were kept in 2 cm³ of 80 % (v/v) methanol in the dark at 4 °C overnight. The extract was centrifuged at 4 000 g and 4 °C for 10 min, and the resulting pellet was re-extracted with another 1 cm³ of 80 % methanol as described above. The supernatants were dried by nitrogen gas and the pellet was re-dissolved in 1 cm³ of methanol, then filtered through a 0.45 μm syringe filter. Quantification of hormones by LC-MS/MS was performed as described by Sasaki *et al.* (2015). Raw values for ABA levels were standardized by plant mass and extraction volume.

Gene expression analysis: Total RNA was extracted from tobacco seeds, and the first strand cDNA was synthesized using a *PrimeScript* RT reagent kit with a gDNA eraser (*Takara*, Dalian, China) according to the manufacturer's instructions. Real-time quantitative PCR was performed as described previously (Zhang *et al.* 2016). The tobacco *actin* gene (*NtActin*) was used as a reference gene. Three independent biological replicates and three technical replicates for each sample were used. The primers are listed in Table 1 Suppl.

Determination of chlorophyll content: Chlorophyll content of the WT and transgenic plants was determined spectrophotometrically using 80 % (v/v) acetone as a solvent (Harmutk 1987). The absorbance of the extract was measured at 645 and 663 nm with an *Optizen 5100* UV spectrophotometer (*Shanghai*, China).

Statistical analysis: All data are presented as the means ± standard errors (SEs) from three biological replicates each containing 50 seeds. Statistical significance was calculated by the Student *t*-test. Analyses were performed with the *IBM SPSS Statistics 18.0* software. Figures were created using *SigmaPlot 12.5* (*Systat* software, Germany).

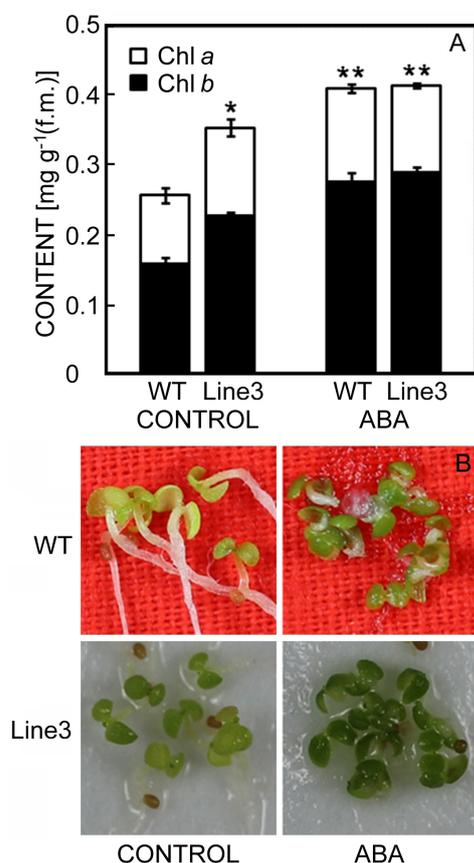


Fig. 4 The effect of *MsZEP* overexpression on chlorophyll (Chl) content in tobacco seedlings. *A* - Chlorophyll content in 28-d-old tobacco seedlings germinated under normal conditions and under 5 μM ABA treatment. *B* - Phenotypes of 28-d-old tobacco seedlings. WT - wild-type tobacco; Line 3 - T₂ *MsZEP*-overexpressing plant line. Means ± SEs, *n* = 3, * and ** - differences significant at *P* ≤ 0.05 and 0.01, respectively.

Results and discussion

Many studies have demonstrated that ABA plays an important role in seed germination (Linkies and Leubner-Metzger 2012, Je *et al.* 2014). Therefore, we hypothesized that also *MsZEP* might play an important role in seed germination. We performed a detailed study of the function of *MsZEP*. Firstly, we examined germination rates of WT and *MsZEP*-overexpressing seeds under normal conditions. We found that the germination rate of WT was significantly higher than of *MsZEP*-overexpressing lines on days 3 and 4 (*P* ≤ 0.01), whereas the germination of WT

was lower than of line 3 but higher than of line 6 on days 5 and 6 ($P \leq 0.01$). These results show that the germination of *MsZEP*-overexpressing seeds was delayed compared with the WT seeds although the final germination percentage of both seeds was similar (Fig. 1A). For reaching 50 % germination, WT seeds needed about 4 d, whereas transgenic seeds needed 4.5 and 5.5 d for line 3 and line 6, respectively; the difference between WT and transgenic lines was significant ($P \leq 0.05$). Under 5 μM ABA treatment, the germination of all seeds was delayed, and the *MsZEP*-overexpressing seeds still germinated slower compared with WT seeds. The germination rate of WT was significantly higher than *MsZEP*-overexpressing lines between days 4 and 6 ($P \leq 0.05$). Wild type seeds reached 50 % germination after around 6 d, whereas overexpressing lines after 6.2 d (Fig. 1B).

It is known that ABA promotes seed dormancy and represses seed germination (Holdsworth *et al.* 2008). Because of the important roles of ZEP in ABA biosynthesis, a higher ABA content was detected in the leaves of *MsZEP*-overexpressing tobacco than in WT (Zhang *et al.* 2016). To investigate whether the delayed germination of *MsZEP*-overexpressing seeds was induced by altered ABA content, ABA content in dry and imbibed seeds was quantified (Fig. 2). Content of ABA in *MsZEP*-overexpressing dry seeds was significantly higher than in WT ($P \leq 0.01$). This is consistent with the study showing that the overexpressing *NiZEP* gene in tobacco under the control of the constitutive 35S promoter results in an increase of ABA content in dry seeds and delays seed germination (Frey *et al.* 1999). However, overexpressing the *Arabidopsis AtZEP* gene in *N. plumbaginifolia* plants using seed-specific promoters does not change ABA content in mature seeds (Frey *et al.* 2006). Since the 35S promoter could regulate the ZEP gene in all tissues, the higher ABA content in dry seeds could be due to the translocation from maternal tissues, like leaves, to seeds. When seeds were imbibed in water for 8 h, ABA content decreased rapidly in both WT and transgenic lines, and finally there was no difference among them (Fig. 2). Similarly, Frey *et al.* (2006) reported that ABA content decreases rapidly and then remains low and stable in imbibed seeds, and there is no difference between WT and *AtZEP*-overexpressing plants, whereas overexpressing the *NiZEP* gene could restore seed dormancy but only slightly delays seed germination compared to WT. Therefore, the delayed germination of *MsZEP*-overexpressing plants might not be caused by the higher ABA content in dry seeds, which might origin from maternal tissues. It was reported that ABA accumulates firstly in maternal tissues and later in the seed embryo (Groot *et al.* 1991). Furthermore, reciprocal crosses between WT and ABA-deficient genotypes proved that only ABA produced by the embryo itself, and not maternal ABA, is necessary to impose dormancy (Karsen *et al.* 1983). Here, overexpressing the *MsZEP* gene under the control of the 35S promoter may increase the seed embryo ABA content in normal conditions and even under ABA addition, which delayed the germination of transgenic seeds despite the fact that we cannot distinguish between ABA produced by seed embryos and maternal tissues. It

was reported that downstream biosynthetic steps, notably NCED activity, contribute to the ABA synthesis regulation in seed embryos (Lefebvre *et al.* 2006). Therefore, *MsZEP* might indirectly regulate seed germination through downstream steps.

Zeaxanthin epoxidase is the first committed enzyme in the ABA biosynthesis pathway, and overexpression of *NpZEP* increases endogenous ABA content and enhances seed dormancy in tobacco (Cutler and Krochko 1999, Wolters *et al.* 2010). The NCED catalyze the rate-limiting step in the ABA biosynthesis pathway (Nambara and Marion-Poll 2005), and overexpression of *NCED* in plants results in higher ABA content and increased seed dormancy (Thompson *et al.* 2000b, Zhang *et al.* 2014, Tong *et al.* 2017). To determine promoting effects of *MsZEP* on ABA accumulation, we analyzed the effect of *MsZEP* overexpression on the expression of ABA biosynthesis genes in seeds using real-time quantitative PCR. The results show that *MsZEP*-overexpressing seeds were not significantly different from WT except for *ZEP1* in line 3. As concerns *NCED*, the expressions of *NCED6* in *MsZEP*-overexpressing seeds were significantly higher than those in WT, whereas the expression of *NCED1* was lower (Fig. 4A; $P \leq 0.05$ or $P \leq 0.01$). The maintenance of seed dormancy is also affected by the ABA synthesis during seed imbibition (Grappin *et al.* 2000). The increased expression of *NCED6* might contribute to the ABA synthesis in embryos of transgenic seeds during imbibition.

Integrity of DNA and proteome stability play a major role in the germination process (Koorneef *et al.* 2002). *AtLig6* is a major determinant of *Arabidopsis* seed quality and longevity, and phenotypic analysis reveals a delay in the germination of *atlig6* mutants compared with WT lines (Waterworth *et al.* 2010). SOMNUS (SOM) is a negative regulator of seed germination (Kim *et al.* 2008). In this study, the effect of *MsZEP* overexpression on the expression of *Lig6* and *SOM* in seeds was analyzed. The *MsZEP*-overexpression lines exhibited a decreased *Lig6* expression and increased *SOM* expression compared with WT (Fig. 4B; $P \leq 0.05$ or $P \leq 0.01$). These results suggest that *MsZEP* negatively regulating germination might affect seed DNA integrity and proteome stability.

The *MsZEP*-overexpressing seedlings appeared dark green compared to the light green WT seedlings (Fig. 5B) suggesting that overexpression of the *MsZEP* gene may promote plant health. This is consistent with findings that the chlorophyll content of *MsZEP*-overexpressing seedlings was significantly higher than that of WT under normal conditions (Fig. 5A; $P \leq 0.05$), which may be due to the role of ZEP in xanthophyll cycle. Moreover, the chlorophyll content of both plants increased after ABA treatment ($P \leq 0.01$). These results suggest that *MsZEP*-overexpression seedlings contained a higher chlorophyll content, which may be due to the high ABA content.

In summary, we reported that the heterologously overexpressing *MsZEP* gene in tobacco increased ABA content in their seeds and negatively regulated seed germination. Enhanced ABA synthesis in seed embryos and delayed germination might also be related to the increased

NCED6 expression. Also, *MsZEP*-overexpression in tobacco seed affected cell proteome and DNA integrity as indicated by the increased expression of *SOM* and a decreased expression of *Lig6*. Moreover, *MsZEP* enhanced chlorophyll content in transgenic tobacco seedlings due to its function in xanthophyll cycle and ABA biosynthesis.

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