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Influence of exogenous 5-aminolevulinic acid on chlorophyll synthesis and related gene expression in oilseed rape de-etiolated cotyledons under water-deficit stress

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Abstract

5-aminolevulinic acid (ALA) is an essential precursor for the biosynthesis of tetrapyrrols such as heme and chlorophyll (Chl). Previous studies have focused mainly on promotive effects of exogenous ALA on plant growth, while regulatory mechanisms affecting Chl biosynthesis have been only partially discussed. In the present study, the ameliorative role of exogenous ALA was investigated on Chl and endogenous ALA biosynthesis in six-day-old etiolated oilseed rape (*Brassica napus* L.) cotyledons during the de-etiolation stage. We showed that exogenously applied ALA of a low dosage enhanced Chl and ALA accumulation in cotyledons, while 600 μM ALA treatment inhibited the accumulation of Chl and ALA severely. However, the gene expression levels of glutamyl-tRNA reductase (*HEMA*) and glutamate-1-semialdehyde aminotransferase (*GSA*) were not affected under either low or high ALA concentrations. Furthermore, water deficit induced by polyethylene glycol 6000 (PEG) suppressed the Chl and ALA accumulation in cotyledons, while the inhibition was partially alleviated in the cotyledons pretreated with ALA. The decrease in Chl biosynthesis induced by PEG stress was assumed to be related to downregulation of *HEMA* and Mg-chelatase ChlH (*ChlH*), and upregulation of ferrochelatase (*FC*) genes. Moreover, exogenously applied ALA did not show any effect on the expression of Chl synthesis-related genes under the PEG treatment. These results showed a difference in suppressing ALA synthesis due to the high concentration of ALA and PEG. Exogenously applied ALA did not affect the expression of *HEMA* and *GSA*, thus exogenous ALA regulated Chl synthesis not *via* the regulation of transcriptional level in ALA biosynthesis. However, the inhibition in Chl and endogenous ALA accumulation by the PEG treatment may be attributed to downregulation of *HEMA* and *ChlH*, and upregulation of *FC*.

Additional key words: protochlorophyllide; rapeseed; transcript; uroporphyrinogen decarboxylase; water stress.

Introduction

5-aminolevulinic acid (ALA) is an essential precursor in tetrapyrrole biosynthesis. Treatment of etiolated angiosperm cotyledons with exogenous ALA caused accumulation of protochlorophyllide (Pchlide) (Granick 1959). Enzymes required for Pchlide synthesis are already active and present in nonlimiting amounts in etiolated plant tissues and only the amount and activity of enzymes involved in ALA synthesis limit the synthesis rate (Granick 1959, Papenbrock and Grimm 2001). The reduction of Pchlide to chlorophyllide is catalyzed by the light-dependent NADPH:Pchlide oxidoreductase (POR) that requires light for its catalysis activity (Schoefs and Franck 2003, Masuda

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Abbreviations: ALA – 5-aminolevulinic acid; Chl – chlorophyll; *ChlH* – gene encoding Mg-chelatase ChlH subunit; *FC* – gene encoding ferrochelatase; FLU – fluorescent; FM – fresh mass; GluTR – glutamyl-tRNA reductase; *GSA* – gene encoding glutamate-1-semialdehyde aminotransferase; *HEMA* – gene encoding glutamyl-tRNA reductase; Lhcb – LHC protein of PSII type III chlorophyll *a*/*b*-binding protein; MgCh – Mg-chelatase; Pchlide – protochlorophyllide; PEG – polyethylene glycol; POR – protochlorophyllide oxidoreductase; qRT-PCR – quantitative real-time PCR; *URO* – gene encoding uroporphyrinogen decarboxylase.

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2008). In addition to light, Chl biosynthesis is also affected by other environmental factors, such as low and high temperature, and oxidative stress (Aarti *et al.* 2006, Mohanty *et al.* 2006). The inhibition of Chl biosynthesis would cause the instability of the pigment-protein complexes and lead ultimately to reduced photosynthesis.

In higher plants, the formation of ALA is the first committed step in the tetrapyrrole biosynthesis leading to the end products, such as Chl, heme, and siroheme, and it is subjected to multiple regulatory mechanisms (Beale 1999, Tanaka and Tanaka 2007). During ALA synthesis, glutamyl-tRNA reduction is most important in terms of regulation (Tanaka and Tanaka 2007). Glutamyl-tRNA reductase (GluTR) is encoded by *HEMA* gene family in higher plants. These *HEMA* genes showed different expression patterns. It has been reported that *HEMA1* is primarily expressed in photosynthetic tissues and its expression is induced by light (Matsumoto *et al.* 2004), while *HEMA2* expression is mainly in nonphotosynthetic organs and is not light-inducible (Tanaka *et al.* 1996, 1997). The GluTR activity is also controlled through feedback regulation by the end product such as heme and Pchlide (Meskauskiene *et al.* 2001, Goslings *et al.* 2004). When the intermediate products of the Chl branch are excessively accumulated, the chloroplast membrane protein FLU would suppress the GluTR1 activity (Meskauskiene *et al.* 2001).

Oilseed rape (*Brassica napus* L.) is one of major oilseed crops and the most important source of dietary lipids in China. It is often subjected to abiotic stress, such as salinity, drought, and low temperature, which limit the yield of oilseed rape. Low dosages of ALA has been found to enhance the Chl accumulation in *Brassica* under both normal and abiotic stress conditions (Memon *et al.* 2009, Naeem *et al.* 2012, Ali *et al.* 2013), while application of high concentrations of ALA suppresses the Chl accumulation (Hotta *et al.* 1997). However, the regulatory mechanisms are yet to be elucidated. In the present study, therefore, the etiolated cotyledons were used to study the effects of exogenous ALA on endogenous ALA accumulation, Chl contents, and the expressions of genes involved in ALA formation and Chl biosynthesis during de-etiolation in oilseed rape. In addition, the effects of exogenous ALA and water-deficit stress on the transcript levels of genes in related to Chl biosynthesis were also investigated.

Materials and methods

Plant material and growth conditions: Healthy seeds of rapeseed (*B. napus* L. cv. ZS 758) were obtained from the College of Agriculture and Biotechnology, Zhejiang University. Seeds were washed with distilled water and air-dried before use and then sowed in germination boxes with double filter paper to which half-strength Hoagland solution was added (Arnon and Hoagland 1940). The composition of Hoagland nutrient solution was as follows (in µM): 3,000 KNO_3; 2,000 $Ca(NO_3)_2 \cdot 4H_2O$; 1,000 $MgSO_4 \cdot 7H_2O$, 10 KH_2PO_4, 12 $FeC_6H_6O_7$, 500 H_3BO_3, 800 $ZnSO_4 \cdot 7H_2O$, 50 $MnCl_2$, 300 $CuSO_4 \cdot 5H_2O$, and 100 Na_2MoO_4. The seeds were then allowed to germinate for 6 d in a growth chamber at 25°C under complete darkness. Then, the cotyledons were excised under dim green light and subjected to further treatments. The temperature was maintained at 25°C throughout the experiments.

Exogenous ALA treatments: Excised etiolated rapeseed cotyledons were placed by adaxial side up on filter paper wetted with different concentrations of ALA (0, 0.06, 0.6, 6, 60, and 600 µM) for 12 h in complete darkness. Then samples were divided into two groups: one was for ALA determination and the other one was exposed to light [100 µmol(photon) m^{-2} s^{-1}] for 24 h in order to detect a Chl content and gene expression.

For water-deficit treatment, the excised etiolated cotyledons were placed adaxial side up on filter paper wetted with ALA (0 and 0.6 µM) for 12 h in complete darkness. Then cotyledons were transferred onto filter paper wetted with 30% polyethylene glycol 6000 (PEG 6000) solution or water for 4 h in dark. These cotyledons were also divided into two groups: one was for ALA determination and the other one was exposed to light [100 µmol(photon) m^{-2} s^{-1}] for additional 24 h at 25°C in order to detect Chl contents and gene expression.

Chl measurement: The total Chl content of cotyledons was extracted with the 80% buffered aqueous acetone containing 2.5 mM sodium phosphate buffer (pH 7.8). The absorbance of the extracts was determined by a spectrophotometer (*UV-2550*, *Shimadzu*, Japan) at 646.6 and 663.6 nm, and the content of Chl was calculated according to Porra *et al.* (1989) using the formula: Chl [mg g^{-1}] = $(17.76A_{646.6} + 7.34A_{663.6}) \times V/1{,}000w$, where $A_{646.6}$ represented the absorbance at 646.6 nm; $A_{663.6}$ represented the absorbance at 663.6 nm; V represented the total volume of an extraction solution [ml]; and w represented the mass of cotyledons [g].

Determination of ALA-synthesizing activity: The cotyledons for the ALA assay were exposed to low light [30 µmol(photon) m^{-2} s^{-1}] for 2 h, and then incubated in 60 mM levulinic acid (*Aldrich*, MO, USA) in 50 mM phosphate buffer, pH 6.0, for 0 and 3 h to determine the ALA content. Samples were homogenized in a prechilled mortar and pestle in 2 ml of 1 M sodium acetate buffer (pH 4.6) and centrifuged at 12,000 × *g* for 10 min at 4°C. Supernatant was taken for the ALA assay according to Tewari and Tripathy (1998). Net ALA synthesis was expressed as the ALA content at 0 h subtracted from the ALA content at 3 h. ALA synthesizing activity was expressed as net ALA accumulation [g^{-}1(FM)] during three hours.

RNA isolation and quantitative real-time PCR: Total RNA was extracted from rapeseed cotyledons using Trizol according to the supplier's recommendation. Residual DNA was removed with DNase I (*Fermentas*, ON, Canada). One microgram of total RNA was reverse transcribed with *RevertAid First Strand cDNA Synthesis* Kit (*Fermentas*, ON, Canada). The gene-specific primers for quantitative real-time PCR amplifications were designed with *Prime Premier 5* based on EST or CDS sequences. The primers used were as follows:

GSA (AF366293) F 5'-CTGAGACGATGAAGAAAGGAACAAGC-3'; R 5'-GAGCGGTCAAAGTATCCGAAGTAGC-3',
HEMA (CD825288) F 5'-CCTGTTGAGATGCGTGAGAA-3'; R 5'-CCCACTTGTCTTTGACATCC-3',
URO (DY023916) F 5'-AAGTGGATCAGGAGGTTTGC-3'; R 5'-CCCCAGGTTCAGAATGTGCT-3',
ChlH (BQ704426) F 5'-CCCAGTAGCCAGCGACCATT-3'; R 5'-TGACTCTGACCCGACGAACC-3',
FC (ES910832) F 5'-AGGGCTCCTAAAAGCAAGGAA-3'; R 5'-TGCCAATAACGCATGCCAACA-3',
Lhcb3 (X61608) F 5'-GCCCAAAGCATTTTAGCCGTCC-3'; R 5'-GTCATTGCCTTCACCAACACCATC-3',
Actin (GQ339782) F 5'-GCTGACCGTATGAGCAAAGA-3'; R 5'-ACGATGGATGGACCCG-3'.

Quantitative real-time PCR was performed using the *Mx3005P* QPCR system (*Agilent*, CA, USA). PCRs were performed using the *Thunderbird SYBR* qPCR Mix (*Toyobo*, Osaka, Japan). The PCR conditions consisted of denaturation at 95°C for 1 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 58°C for 35 s, and extension at 72°C for 45 s. Expression of the target genes was normalized relative to the expression of the housekeeping gene *actin* to minimize sample variations. The quantification of mRNA levels was based on the method of Livak and Schmittgen (2001).

Statistical analysis: All the experiments were performed with three replicates and results were expressed as mean ± SD. Statistical analysis was performed using *SAS 9.1* software. Analysis of variance (*ANOVA*) was followed by the *Tukey*'s test in order to ascertain significant differences of the means between treatments. Results were considered significant at $p<0.05$.

Results

Chl and endogenous ALA synthesis: The effects of exogenous ALA on Chl accumulation in etiolated cotyledons over time were investigated up to 24 h (Fig. 1*A*). Application of ALA at 600 µM inhibited the accumulation of Chl in the cotyledons during 24 h under continuous light. However, the contents of Chl in the cotyledons treated with 0–60 µM ALA increased rapidly after being exposed to low light. After 24 h of the light treatment, the Chl contents of the cotyledons treated with exogenous ALA at 0.06, 0.6, and 6 µM were 384.9, 416.2, and 403.9 µg g^{-1}(FM), increasing by 19, 28, and 25%, respectively, as compared with that of treated with 0 µM ALA.

Moreover, it was found that the exogenous application of ALA at the higher concentration (600 µM) inhibited the accumulation rate of endogenous ALA severely in the etiolated cotyledons; it declined by 78% compared with control conditions. However, the low concentrations of exogenous ALA improved the accumulation rate of

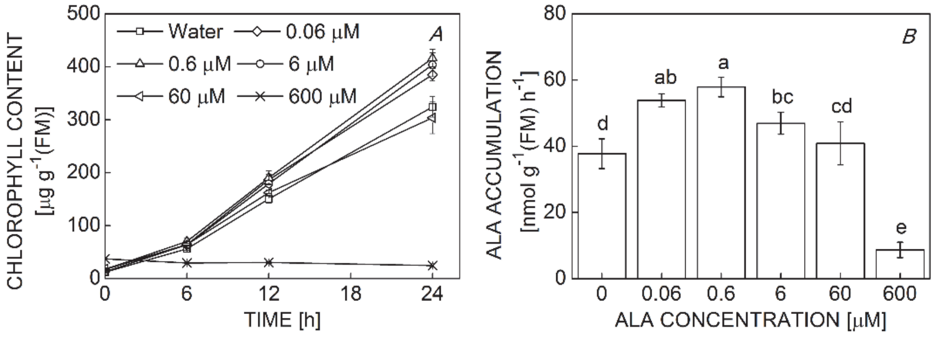

Fig. 1. Effects of different concentrations of exogeous 5-aminolevulinic acid (ALA) on (*A*) time-course of chlorophyll content, and (*B*) ALA synthesizing activity in rapeseed cotyledons. Excised etiolated cotyledons were subjected to ALA treatment (0, 0.06, 0.6, 6, 60, and 600 µM) for 12 h in complete darkness. Subsequently, (*A*) they were transferred to low light and the chlorophyll content measured periodically up to 24 h; (*B*) they were exposed to low light for 2 h, and then incubated in 60 mM levulinic acid for 3 h. ALA synthesizing activity was measured as ALA accumulation during 3 h. Values are the means of three replicates with SD shown by *vertical bars*. Columns denoted by *the same letter* are not significantly different according to *Tukey*'s test ($p<0.05$).

endogenous ALA, which increased by 43, 54, and 24% at 0.06, 0.6, and 6 μM, respectively (Fig. 1*B*). The synthesis rate of endogenous ALA in the etiolated cotyledons showed no significant difference between 60 and 0 μM ALA applications. In order to determine a reason behind the different endogenous ALA-synthesizing activity under different exogenous ALA concentrations, the transcript levels of genes governing ALA biosynthesis, *i.e.*, *GSA* (gene encoding glutamate-1-semialdehyde aminotransferase) and *HEMA* were monitored by means of quantitative real-time PCR analysis in the cotyledons. Our results showed that the expressions of these two genes were not affected by the different concentrations of ALA, even after 600 μM ALA application (Fig. 2).

Effects on Chl and endogenous ALA synthesis under PEG stress: In the present study, 0.6 μM ALA concentration was selected in order to investigate the effects on the Chl and endogenous ALA synthesis in the etiolated cotyledons under water stress (PEG-6000). The PEG treatment suppressed the Chl accumulation in the cotyledons under light (Fig. 3*A*). After 24-h of the light treatment, the contents of Chl after the PEG treatment decreased by 43%, compared to that with water treatment, while the inhibition was partially alleviated in the cotyledons pretreated with ALA. Similarly, the PEG treatment also inhibited the synthesis of endogenous ALA, which was reduced by 27% compared to that with the water treatment (Fig. 3*B*). The combination of ALA and PEG treatments inctreased the content of endogenous ALA by 19% in the cotyledons compared to that treated by PEG alone. The effects of the exogenous ALA and PEG treatment on expression levels of *GSA* and *HEMA* were also examined. The PEG treatment significantly downregulated these two genes, they were reduced to 34 and 54%, respectively (Fig. 4). However, the gene transcript levels were not affected by exogenous ALA under the PEG treatment.

Effects on expression of Chl biosynthetic genes: In addition to *GSA* and *HEMA*, the transcript levels of other genes involved in Chl synthesis, including uroporphyrinogen decarboxylase (*URO*), *ChlH*, *FC*, and LHC protein of PSII type III chlorophyll *a*/*b*-binding protein (*Lhcb*) were also examined (Fig. 5). PEG stress significantly inhibited the expression of *URO*, *ChlH*, and *Lhcb*, which was reduced to 46, 43, and 23%, respectively. However, the expression of *FC* was induced to 2.3 fold by the PEG treatment. Application of exogenous ALA alone did not affect the transcript levels of *URO*, *ChlH*, and *Lhcb*, while it induced the expression of *FC*. The ALA treatment inhibited the expression levels of *FC* and *Lhcb* under PEG stress (Fig. 5).

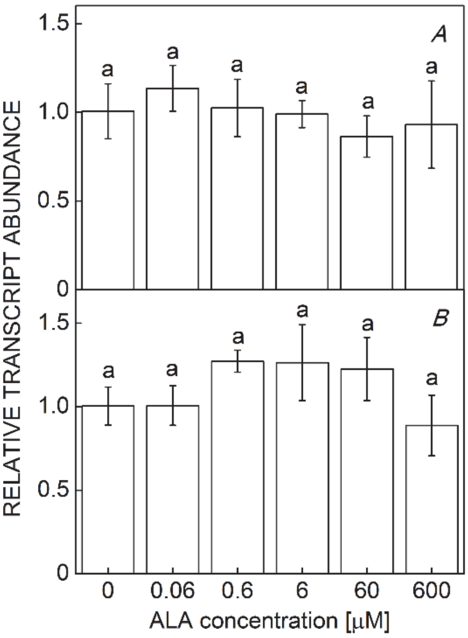

Fig. 2. Effects of different concentrations of exogeous 5-aminolevulinic acid (ALA) on the steady-state transcript level of genes encoding glutamate-1-semialdehyde aminotransferase (*GSA*) (*A*) and glutamyl-tRNA reductase (*HEMA*) (*B*) in rapeseed cotyledons after being exposed to light for 24 h. Excised etiolated cotyledons were subjected to ALA treatment (0, 0.06, 0.6, 6, and 60 μM) for 12 h in complete darkness. Subsequently, these were transferred to low light for 24 h to determine the expression levels of *GSA* and *HEMA*. Values are the means of three replicates with SD shown by *vertical bars*. Columns denoted by *the same letter* are not significantly different according to *Tukey*'s test (p<0.05).

Discussion

Chl biosynthesis may be regulated at the level of ALA formation during illumination (Beale and Castelfranco 1974, Ilag *et al.* 1994, Aarti *et al.* 2007). However, most studies used high concentrations, *i.e.*, 5–40 mM, which could induce oxidative stress (Granick 1959, Sisler and Klein 1963, Sundqvist 1969). It has been found that exogenously applied low concentrations of ALA enhanced the Chl synthesis in plants (Hotta *et al.* 1997, Memon *et al.* 2009). In the present study, we found that the Chl contents in the etiolated cotyledons were not continuously enhanced with increasing exogenous ALA concentrations (Fig. 1). The cotyledons after the application of the high concentration of ALA (600 μM) barely accumulated Chl after being exposed to low light for 24 h (Fig. 1*A*). Similar findings were also observed by Hotta *et al.* (1997), who reported that promotion of Chl contents by exogenous ALA was greatly concentration depended. ALA significantly increased the total Chl content in horseradish cultured at the concentration of 0.06 μM, but regressed at 60 μM. The application of the higher concentration gradually caused growth retardation, and the rice seedlings were clearly damaged after being treated with 600 μM (Hotta *et al.* 1997). Kittsteiner *et al.* (1991) also reported that excess ALA affected chloroplast development and reduced the

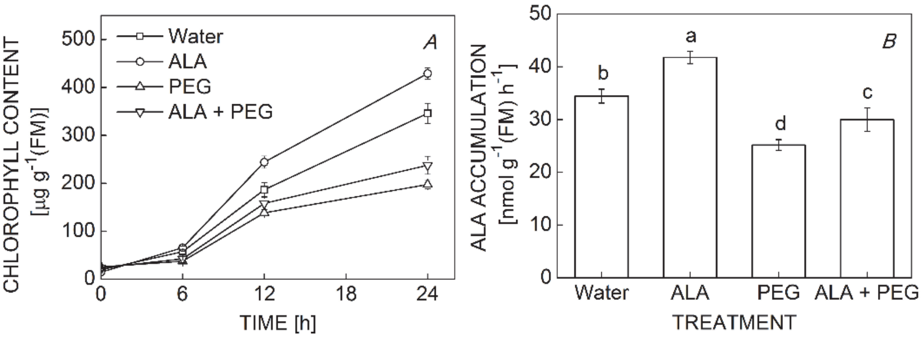

Fig. 3. Effects of exogeous 5-aminolevulinic acid (ALA) and water-deficit stress induced by PEG on (*A*) time-course of Chl content, and (*B*) ALA synthesizing activity in rapeseed cotyledons. Excised etiolated cotyledons were subjected to ALA treatment (0 and 0.6 μM) for 12 h and then transferred onto filter paper wetted with 30% PEG 6000 solution or water for 4 h in complete darkness. Subsequently, (*A*) these were transferred to low light and the Chl content measured periodically up to 24 h; (*B*) these were exposed to low light for 2 h, and then incubated in 60 mM levulinic acid for 3 h. ALA synthesizing activity was measured as ALA accumulation during 3 h. Values are the means of three replicates with SD shown by *vertical bars*. Columns denoted by *the same letter* are not significantly different according to *Tukey*'s test ($p<0.05$).

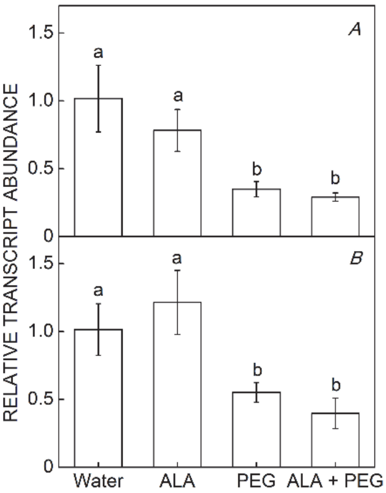

Fig. 4. Effects of exogeous 5-aminolevulinic acid (ALA) and water-deficit stress induced by PEG on the steady-state transcript level of genes encoding glutamate-1-semialdehyde aminotransferase (*GSA*) (*A*) and glutamyl-tRNA reductase (*HEMA*) (*B*) in rapeseed cotyledons. Excised etiolated cotyledons were subjected to ALA treatment (0 and 0.6 μM) for 12 h and then transferred onto filter paper wetted with 30% PEG 6000 solution or water for 4 h in complete darkness. Subsequently, these were transferred to low light for 24 h to determine the expression levels of *GSA* and *HEMA*. Values are the means of three replicates with SD shown by *vertical bars*. Columns denoted by *the same letter* are not significantly different according to *Tukey*'s test ($p<0.05$).

rate of greening after continuous illumination in dark-grown cress seedlings. High concentrations of ALA (10–20 mM) showed also negative effects on Chl accumulation in etiolated leaves of gymnosperms, such as *Larix decidua* Mill. and *Picea abies* (L.) Karst, after illumination (Pavlovič *et al.* 2009, Maximová and Slováková 2014). These results suggested that exogenous ALA acted as a growth regulator on the Chl content in plants at low concentrations (Hotta *et al.* 1997, Liu *et al.* 2011).

The synthesizing activity of endogenous ALA significantly affects the accumulation of Chl since the formation of ALA is the committed step in Chl biosynthesis (Tanaka and Tanaka 2007). In the present study, low concentrations of exogenous ALA enhanced the endogenous ALA biosynthesis, while the high concentrations of ALA impaired the ALA-biosynthetic activity (Fig. 1*B*), which suggested that the promotive effects of exogenous ALA on Chl accumulation might be partly attributed to enhancement of the ALA-synthesizing activity. Two principal mechanisms regulating endogenous ALA biosynthesis have been reported. One involves heme, which acts as a feedback inhibitor of GluTR and regulates Chl biosynthesis (Pontoppidan *et al.* 1994). The other involves FLU protein, which also interacts with GluTR and suppresses its activity when Pchlide accumulates excessively (Meskauskiene *et al.* 2001). The transcript levels of *GSA* and *HEMA* did not change when treated by different concentrations of exogenous ALA, even though the 600 μM ALA treatment inhibited the synthesis of ALA severely (Figs. 1, 2). Similarly, high light impaired the ALA-biosynthetic activity severely without inhibiting the transcript levels of *HEMA* and *GSA*. The inhibition of ALA synthesis by high light was partly due to the decrease in protein of GluTR in de-etiolated cucumber cotyledons (Aarti *et al.* 2007). The excessive exogenous ALA could lead to the accumulation of intermediates of Chl biosynthesis, such as Pchlide (Castelfranco and Jones 1975). The accumulated Pchlide regulated FLU protein to suppress GluTR activity. On the other hand, the excessive intermediates of Chl biosynthesis can interact with molecular oxygen to generate reactive oxygen species (Aarti *et al.* 2006), which might be also responsible for the inhibition of ALA synthesis in cotyledons exposed to high doses of exogenous ALA treatment (Aarti *et al.* 2007).

However, the transcript levels of *HEMA* and *GSA* were inhibited by the PEG treatment (Fig. 4). The expression of *Lhcb* was also suppressed by the PEG treatment (Fig. 5*D*).

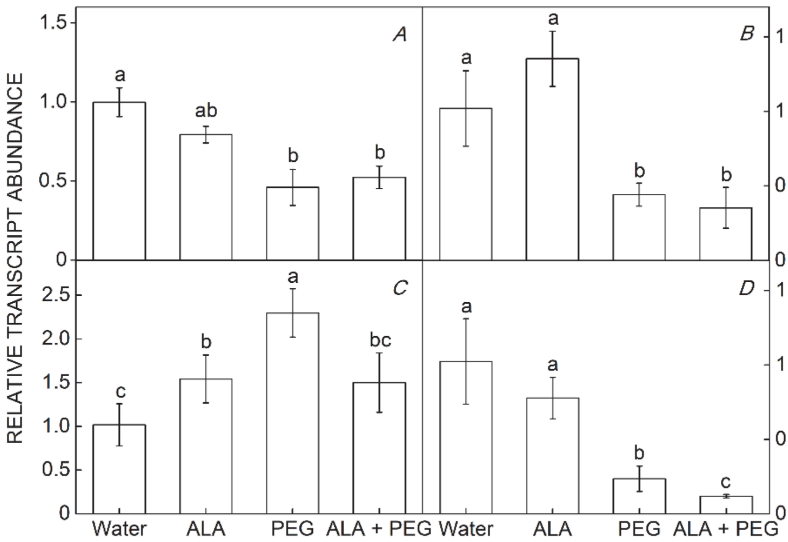

Fig. 5. Effects of exogeous 5-aminolevulinic acid (ALA) and water-deficit stress induced by PEG on the steady-state transcript level of genes involved in Chl synthesis in rapeseed cotyledons. Excised etiolated cotyledons were subjected to ALA treatment (0 and 0.6 μM) for 12 h in complete darkness and then transferred onto filter paper wetted with 30% PEG 6000 solution or water for 4 h in dark. Subsequently, these were transferred to low light for 24 h to determine the expression levels of genes encoding uroporphyrinogen decarboxylase (*URO*) (*A*), Mg-chelatase ChlH subunit (*ChlH*) (*B*), ferrochelatase (*FC*) (*C*), and LHC protein of PSII type III Chl *a*/*b*-binding protein (*Lhcb*) (*D*). Values are the means of three replicates with SD shown by *vertical bars*. Columns denoted by *the same letter* are not significantly different according to *Tukey*'s test (p<0.05).

Lhcb is involved in maintaining stability of thylakoid membrane (Tanaka and Tanaka 2007). The significant inhibition of *Lhcb* expression suggested that the chloroplast might be destructed. In the present study, the photosynthetic apparatus might be impaired by water-deficit stress induced by the PEG treatment, which could contribute to the decline in expressions of *HEMA* and *GSA*. Another regulatory point during Chl synthesis is the branching point of heme and Chl biosynthesis. At this point, Mg-chelatase catalyzes the insertion of the Mg^{2+} into protoporphyrin IX, ultimately to form Chl, while ferrochelatase inserts Fe^{2+} into protoporphyrin IX to form protoheme. The PEG treatment induced the expression of *FC*, while inhibited the transcript levels of *ChlH* (Fig. 5*B*,*C*). The enhanced expression of *FC* leads to increased metabolic flux into heme and the heme biosynthesis is transiently upregulated during stress (Kim *et al.* 2014). We assumed that the PEG treatment might enhance heme biosynthesis, while the increase in heme causes a feedback inhibition in GluTR activity. The decrease in *ChlH* expression inhibited the activity of Chl biosynthesis branch directly. These results indicated that the mechanisms of ALA synthesis inhibition caused by the high concentrations of ALA and PEG might be different: The PEG stress downregulated the expression of *HEMA* and *GSA*, while high dosages of ALA inhibited the GluTR activity not *via* transcriptional regulation.

The other inactive enzymes involved in Chl synthesis could also suppress the synthesis of Chl (Tewari and Tripathy 1998, 1999). The expressions of the genes related to Chl synthesis were significantly regulated by temperature stress. Low-temperature stress downregulated the expression of *URO* accompanied with the decrease in URO activity (Mohanty *et al.* 2006). In the present study, the PEG stress downregulated the expression of *URO*, while the ALA treatment did not show any change (Fig. 5*A*). Moreover, the expression of *Lhcb* was severely inhibited by the PEG treatment (Fig. 5*D*). The expression pattern of *HEMA* was strongly correlated with those of *Lhcb*, which indicated that the *HEMA* expression reflects the cellular demand for Chl synthesis. The coordinated synthesis of Chl and the Chl *a*/*b*-binding proteins is critical to the development of functional light-harvesting complexes during de-etiolation (McCormac and Terry 2002, Matsumoto *et al.* 2004).

In conclusion, the mechanisms of inhibition in Chl synthesis by the high concentration of ALA and PEG were different. ALA at the high concentration suppressed Chl synthesis without affecting the expression of *HEMA* and *GSA*. However, the PEG treatment inhibited Chl synthesis and endogenous ALA accumulation, which might be attributed to the downregulation of *HEMA* and *ChlH*, and the upregulation of *FC*. Low concentrations of ALA enhanced Chl synthesis through improving the endogenous ALA biosynthesis; however, the expression levels of *HEMA* and *GSA* were also not affected. Thus, exogenous ALA did not affect endogenous ALA synthesis at the transcript level.

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