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BRIEF COMMUNICATION

The accumulation of chlorophyll and changes of ATP during greening of etiolated *Arabidopsis* seedlings are mediated by nitric oxide

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Abstract

Seedling greening upon irradiance is essential for the survival of plant after germination. Here, we studied the role of nitric oxide (NO) in regulating the accumulation of chlorophyll during greening of *Arabidopsis* seedlings, and we also investigated the changes of ATP in the chloroplast and cytoplasm during greening by using a fluorescent protein sensor (Ateam 1.03-nD/nA) based on fluorescence resonance energy transfer (FRET) and the effects of NO on the changes of ATP content. The results showed that the content of NO, chlorophyll, and ATP in the chloroplast and cytoplasm increased with the increase of greening time. L-NAME, an inhibitor of NO, not only decreased the accumulation of chlorophyll content but also reduced the ATP content in the chloroplast and cytoplasm during the greening of *Arabidopsis* seedlings. Therefore, these experiments indicate that the accumulation of chlorophyll and changes of ATP during greening of etiolated *Arabidopsis* seedlings are mediated by nitric oxide.

Keywords: *Arabidopsis thaliana*, ATeam 1.03-nD/nA, ATP, chlorophyll, etiolation, greening, nitric oxide.

Seedling greening upon irradiance is essential for the survival of plant emerging after germination (Pipitone *et al*. 2021). During seedling greening, seedlings undergo changes in morphogenesis, such as fast growth of the hypocotyl (Nemhauser 2008, Armarego-Marriott *et al*. 2020). In fact, before those visual alterations appear, many physiological changes have occurred during the early stage of greening. Chlorophyll biosynthesis is a pivotal event during the greening of etiolated seedlings (Jedynak *et al*. 2022). This process is dependent on the reduction of protochlorophyllide to chlorophyllide and is accomplished *via* a complex biosynthetic pathway (Zhang *et al*. 2018). Another important event during seedling greening is the change in ATP production in the plant cells (Chadee *et al*. 2021). During the greening, once the biogenesis of thylakoid membranes is completed, the ATP production in the chloroplast occurs and chloroplasts begin to perform CO_2 assimilation for further development of the plants (Allen *et al*. 2011). This might inevitably decrease the dependency of chloroplasts on the ATP that is transported from the cytosol. Thus, the content of the ATP in the chloroplasts could be gradually changed with the greening.

Signaling molecules of plants, such as Ca^{2+}, H_2O_2, and NO, are known to play important roles in regulating the growth and development of plants and the responses to environmental stresses (Lang *et al*. 2020). On one hand, the mechanism for the regulation of seedlings greening by signaling molecules is still poorly studied. On the other hand, the traditional methods of measuring ATP content seem difficult to evaluate its content in different cellular

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Abbreviations: Chl - chlorophyll, FRET - fluorescence resonance energy transfer, L-NAME - L-nitroarginine methyl ester.
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compartments, since there is cross contamination of ATP from different organelles when the samples are broken-down and organelles are isolated by centrifugation or other methods (Rajendran *et al.* 2016). In recent years, based on the fluorescence resonance energy transfer (FRET), De Col *et al*. (2017) established the *Arabidopsis* lines expressing the ATeam1.03-nD/nA fluorescent protein sensor based on the FRET to indicate the change of ATP content in the cytosol and the chloroplasts (De Col *et al*. 2017). The FRET occurred when the acceptor fluorescent protein (cp173Venus) is excited by the emission energy of the donor fluorescent protein (mseCFP). The change of the FRET signal, *i.e*., the Venus/CFP ratio, can reflect the content of ATP (De Col *et al*. 2017). In the present study, by using this method, we demonstrate that the accumulation of chlorophyll and changes of ATP levels in the cytosol and the chloroplasts during greening of etiolated *Arabidopsis* seedlings are mediated by NO.

The seeds of *Arabidopsis thaliana* expressing ATeam1.03-nD/nA in the cytosol (cyt-ATeam) and the chloroplasts (cp-ATeam) were kindly provided by Markus Schwarzländer (Institute of Crop Science and Resource Conservation, University of Bonn, Germany). The seeds of cyt-ATeam, cp-ATeam, and wild type (Columbia, Col-0) were sterilized with 3% NaClO for 5 min and then were rinsed with sterile water 3 times. The sterilized seeds were grown on semi-strength Murashige and Skoog (MS) medium containing 1% (m/v) sucrose and 0.5% (m/v) agarose. The plates were placed vertically under dark conditions at 4°C for 72 h and then were exposed to white light for 16 h to stimulate germination. After then, the plates with the germinated seeds were transferred to complete darkness and cultured vertically at 23°C for 120 h. For the de-etiolation of the seedlings, the etiolated seedlings were transferred to the condition of a 16-h photoperiod with an irradiance of 100 μmol m^{-2} s^{-1} and were cultured for 120 h.

Before the end of the dark period of every light-dark cycle during the 120 h of de-etiolation, the seedlings were rinsed with distilled water and transferred to the wells of the 96-well plate. The seedlings were immersed with 1 mM L-nitroarginine methyl ester (L-NAME, a nitric oxide synthase inhibitor) and incubated in darkness at 23°C for 1 h. The seedlings that were immersed in distilled water and incubated under the same condition were used as the controls. After the treatment, the seedlings were used for the measurements of NO content, chlorophyll content, and the Venus/CFP ratio.

The FRET ratio was measured by multiwell plate reader-based fluorimetry as described by De Col *et al.* (2017). The seedlings were placed in the microplate for measurement of the FRET ratio in a *TECAN SPARK* (*Decon Experimental Equipment Co.*, *Ltd.*, Shanghai, China) microplate reader. ATeam1.03-nD/nA was excited at 435 nm and emissions were collected at 483 nm (for the mseCFP) and 535 nm (for the cp173Venus), respectively (Sweetlove *et al.* 2007). The ATP content was presented with the Venus/CFP ratio (De Col *et al.* 2017). The emission in the wells withCol-0 plants was subtracted from that of ATeam1.03-nD/nA-expressing plants to correct for background fluorescence.

Chlorophyll content was measured as described by Zhang *et al*. (2016). The leaves were ground with 80% acetone to allow the tissue to be thoroughly homogenized. The homogenate was centrifuged at 7 104× *g* for 3 min to obtain the supernatant. The absorbance of the chlorophyll was recorded at 663 and 645 nm, respectively, by a *721* spectrophotometer. The 80% acetone was used as a blank control.

The content of NO was detected with NO fluorescent probe, 3-amino, 4-aminomethyl-2',7'-difluorescein diacetate (DAF-FM DA) (Qiao *et al*. 2015). Before the treatment with L-NAME, the seedlings were immersed with 5 μM·DAF-FM DA (pH 7.2) (Wu and Wu 2008) and were incubated in darkness for 30 min. After then, the seedlings were rinsed with phosphate-buffered saline (PBS) to remove the excess and unbound dye. The DAF-FM DA fluorescence was measured with a *TECAN SPARK* microplate reader. The seedlings were excited at 495 nm and emissions were collected at 515 nm.

All experiments were done on at least three individual biological replicates, and the results are expressed as mean values with standard deviation (SD). *IBM SPSS Statistics v. 25.0* (SPSS) analysis software was used to conduct one-way *ANOVA* for the obtained data, and the least significant difference (LSD) was used for multiple comparisons; $P < 0.05$ was considered a significant difference.

The changes in morphogenesis of the de-etiolation process were shown in Fig. 1*A*. It was observed that with the increase of greening time, the cotyledons opened and became green. It is known that NO is an important endogenous signal molecule and can regulate seed germination, hypocotyl elongation, and the growth and development of plants (Lozano-Juste and León 2011). With the increase of time of etiolation, NO content was significantly increased (Fig. 1*B*). The NO content peaked at 96 h after the start of de-etiolation, while the NO content was lower at 120 h after the start of de-etiolation, compared to that at 96 h after the start of de-etiolation. Following the increase of NO content, the chlorophyll content also increased (Fig. 1*C*).

We further investigated the effect of etiolation on the ATP content in the cytosol and the chloroplasts by using the cyt-ATeam and cp-ATeam lines (Fig. 1 Suppl.). At 24 h after de-etiolation, the FRET ratio of the cp-ATeam line was about 0.62. With the increase of the time of de-etiolation, the FRET ratio of the cp-ATeam line gradually increased, presenting a similar change trend with that of chlorophyll content (Fig. 1*D*). This observation indicates that the ATP content in the chloroplasts is enhanced during the de-etiolation. This is not surprising, since greening can stimulate chloroplast formation and development, thus endowing the capacity of chloroplasts to produce ATP. At 24 h after the start of the de-etiolation, the FRET ratio of the cyt-ATeam line was about 0.9, while the FRET ratio of the cyt-ATeam line dramatically increased to approximately 2.4 at 48 h after the start of

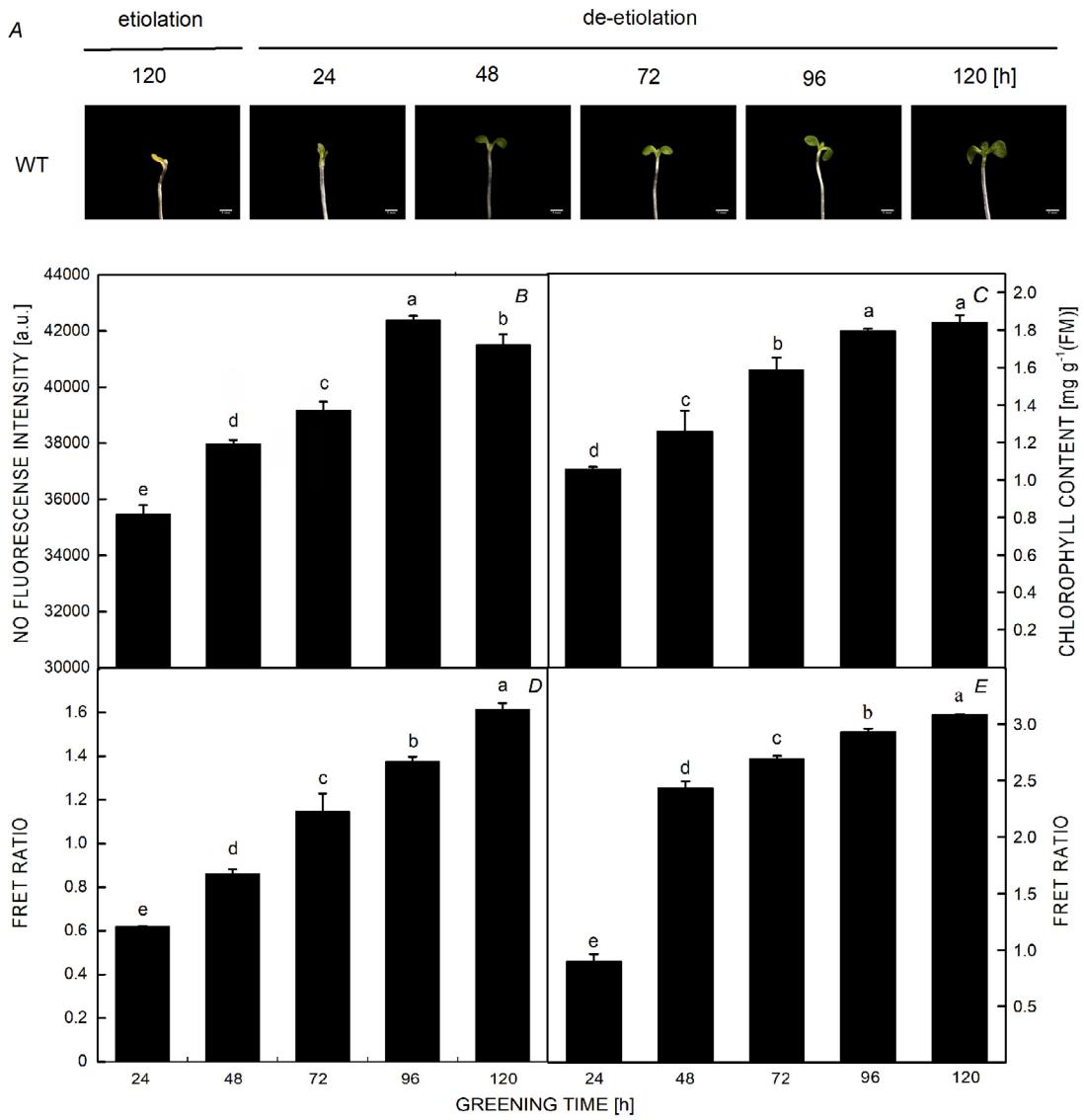

Fig. 1. The changes of morphogenesis (*A*), the intensity of NO fluorescence (*B*), chlorophyll content (*C*), and the FRET ratios of cp ATeam (*D*) and cyt ATeam (*E*) during de-etiolation of the *Arabidopsis* seedlings. Means ± SDs of three individual replications at least. *Different small letters* indicate a significant difference (at $P < 0.05$).

de-etiolation, compared to that at 24 h after the start of de-etiolation. This result showed that the ATP content in the cytoplasm was also enhanced during the de-etiolation, and the ATP content in the cytoplasm was enhanced more dramatically than that in the chloroplasts during the early stage of greening. Previous works showed that there is a major increase in gene expression and protein synthesis during the early stage of greening, particularly in those concerned with chloroplast development (Abbas *et al.* 2015). Theoretically, during the early stage of greening, the ATP needed for the transcription and protein synthesis could be mainly provided by cytoplasm, rather than by the chloroplasts themselves, since the chloroplasts during this stage are still immature. However, with the chloroplast formation and development during the greening, the dependence of the seedlings on the ATP supplied by the cytoplasm could be weakened, which could be reflected by our observation that with the further increase of the time of de-etiolation, the extent of increase of the FRET ratio of cyt-ATeam become moderate (Fig. 1*E*).

The present work further verified whether the observed increase in the chlorophyll content and ATP content in the chloroplasts and the cytosol during de-etiolation could be regulated by NO. L-NAME, a NO synthase inhibitor, was used to investigate such an issue. Application of L-NAME effectively decreased the NO content of the seedlings during de-etiolation (Fig. 2*A*; Fig. 2 Suppl.). Similar to the effect of L-NAME on the NO content of the seedlings during de-etiolation, L-NAME also reduced the chlorophyll accumulation during de-etiolation, suggesting that NO could regulate the biosynthesis of chlorophyll during de-etiolation (Fig. 2*B*; Fig. 2 Suppl.,

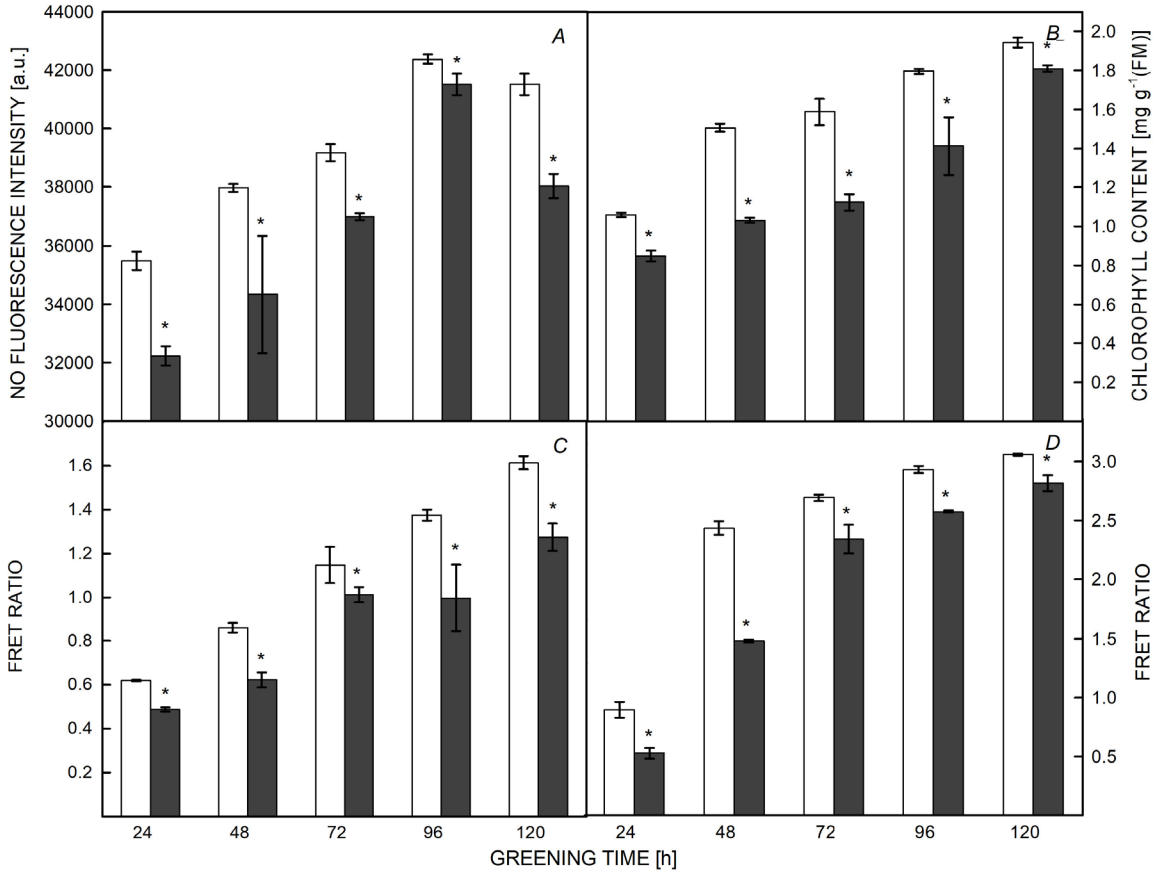

Fig. 2. Effects of L-NAME on NO fluorescence intensity (*A*) and chlorophyll content (*B*) of wild-type seedlings, and the effects of L-NAME on FRET ratio of cp (*C*) and cyt (*D*) ATeam 1.03-nD/nA during de-etiolation. *The white columns* represent the control group and *the black columns* represent the L-NAME processing group. Means ± SDs of three individual replications at least. *Asterisks* indicate a significant difference (at $P < 0.05$).

Fig. 3 Suppl.). This observation is well consistent with the previous reports that the donor of NO can improve chlorophyll content in many species of plants under stressful or non-stressful conditions (Abbas *et al.* 2015, Shang *et al.* 2022). Beligni and Lamattina (2000) proposed that NO could act as a stimulator molecule in plant photomorphogenesis, either dependent on or independent of plant photoreceptors, and thus mediate the accumulation of chlorophyll (Beligni and Lamattina 2000). The work by Melo *et al.* (2016) in the tomato seedlings suggests that NO could promote the chlorophyll content possibly by inhibiting ethylene biosynthesis and inducing auxin accumulation (Melo *et al.* 2016).

It is known that NO can cause a strong inhibition of respiration (Brown and Borutaite 2001, Hou *et al.* 2021). Thus, it is expected that the increase of NO content during greening could inevitably decrease the ATP content. To investigate this issue, we further studied the effect of L-NAME on FRET ratios of the cp-ATeam and cyt-ATeam lines during de-etiolation. However, the results showed that there was a significant decrease in the FRET ratios of either cp-ATeam or cyt-ATeam lines after L-NAME treatment, compared with those without L-NAME treatment. At 24 h after the start of de-etiolation, the FRET ratio of the cp-ATeam line was decreased by about 21% by L-NAME treatment. At 48 h after the start of de-etiolation, the FRET ratio of the cp-ATeam line was dramatically decreased by approximately 27% by L-NAME treatment. At 72, 96, and 120 h after the start of de-etiolation, the FRET ratios of the cp-ATeam line were decreased by about 12, 27, and 21%, respectively, by L-NAME treatment (Fig. 2*C*).

At 24 h after the start of de-etiolation, the FRET ratio of the cyt-ATeam line was dramatically decreased by approximately 41% by L-NAME treatment. At 48 h after the start of de-etiolation, the FRET ratio of the cyt-ATeam line was decreased by about 39% by L-NAME treatment. At 72, 96, and 120 h after the start of de-etiolation, the FRET ratios of the cyt-ATeam line were decreased by about 13, 12, and 8%, respectively, by L-NAME treatment (Fig. 2*D*). These results suggest that NO also plays an important role in positively regulating the ATP content in the cytosol and the chloroplasts during the process of de-etiolation.

On one hand, these observations indicate that the physiological content of NO generated during greening could be lower than the threshold concentration that can inhibit respiration. On the other hand, these observations

also indicate that the physiological increase of NO content seems to be required for the enhancement of the ATP content in the cytosol and the chloroplasts during greening. It is noted that under conditions of hypoxia or flooding, NO and phytoglobin (Pgb) can form a 'Pgb-NO cycle', which can contribute to the recycling of NADH and the synthesis of ATP (Hou *et al.* 2021). It is still unknown whether the 'Pgb-NO cycle' could also play a role in regulating ATP production during greening. But this provides a possible mechanism for the regulation of ATP content in the cytosol and in the chloroplasts by NO during greening. Further work is needed to confirm this conjecture or other possibilities.

Regardless of how complex the mechanism of the regulation of ATP content during greening is, the present work suggests that the accumulation of chlorophyll and changes of ATP content in the cytosol and the chloroplasts during greening of etiolated *Arabidopsis* seedlings are mediated by NO.

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