EFFECT OF DROUGHT STRESS ON PORPHYRIN BIOSYNTHESIS IN RICE SEEDLINGS

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ABSTRACT

Porphyrins play vital roles in various biological processes. This study focused on porphyrin biosynthesis control under drought stress in rice plant. The results showed that in response to drought treatment by water withholding, the level of porphyrin intermediates greatly decreased after 36 h of treatment whereas final products (chlorophyll and heme) content just slightly reduced after 60 h of drought stress. The activity of some key enzymes in porphyrin pathway, including ALA-synthesizing capacity, PPO activity, Mg-chelatase activity, Fe-chelatase activity reduced together with the down-regulation of porphyrin biosynthetic genes and nuclear-encoded photosynthetic genes, especially at 48 and 60 h after treatment. It indicated a sensitivity of porphyrin pathway to drought stress. And it also demonstrated a tight control of porphyrin biosynthesis in order to prevent the accumulation of toxic metabolic intermediates by down-regulation of their biosynthesis under drought condition.

Keywords: Chlorophyll, drought stress, heme, porphyrin biosynthesis, rice plant.

Abbreviates: ALA: 5-aminolevulinic acid, *ALAD*: ALA dehydratase, *CHLD*: D-subunit of Mg-chelatase, *CHLH*: H-subunit of Mg-chelatase, *CHLI*: I-subunit of Mg-chelatase, *FC*: ferrochelatase, *GSA:* glutamate 1-semialdehyde aminotransferase, *HEMA*: glutamyl-tRNA reductase, MgProto: Mg-protoporphyrin IX; MgProto ME: Mg-protoporphyrin IX monomethylester; Pchlide: protochlorophyllide, *PORB*: protochlorophyllide oxidoreductase B, *PPO*: protoporphyrinogen oxidase, Proto IX: protoporphyrin IX, ROS: reactive oxygen species.

Ảnh hưởng của hạn tới sinh tổng hợp porphyrin ở cây lúa

TÓM TẮT

Quá trình sinh tổng hợp porphyrin đóng vai trò quan trọng trong các hoạt động trao đổi chất diễn ra trong cơ thể sinh vật. Nghiên cứu của chúng tôi tập trung vào quá trình sinh tổng hợp porphyrin trong điều kiện hạn trên cây lúa. Kết quả cho thấy hàm lượng các chất trung gian trong quá trình sinh tổng hợp porphyrin giảm mạnh sau 36 h ngừng tưới nước, trong khi đó hàm lượng hai sản phẩm cuối của quá trình sinh tổng hợp porphyrin là diệp lục và heme thì chỉ giảm ít sau 60 h xử lý hạn. Hoạt động của các enzyme chìa khóa trong con đường này bao gồm khả năng sinh tổng hợp ALA, hoạt động của PPO, Mg-chelatase, Fe-chelatase cũng giảm cùng với sự giảm biểu hiện của các gen trong quá trình sinh tổng hợp porphyrin và một số gen liên quan tới hoạt động quang hợp được mã hóa trong nhân, đặc biệt ở 48 và 60 h sau khi xử lý hạn. Từ những kết quả thu được cho thấy quá trình sinh tổng hợp porphyrin bị ảnh hưởng rất lớn trong điều kiện hạn. Kết quả nghiên cứu cũng chứng tỏ rằng có một cơ chế phối hợp điều khiển chặt chẽ để ngăn ngừa sự tích lũy các sản phẩm trung gian trong quá trình sinh tổng hợp porphyrin trong điều kiện hạn.

Từ khóa: Cây lúa, diệp lục, heme, khô hạn, sinh tổng hợp porphyrin.

1. INTRODUCTION

Porphyrins have important function in various biological processes, such as photosynthesis, respiration, morphogenesis, and detoxification. Higher plants produce four classes of porphyrins which consist of chlorophyll and heme (Fig. 1). The porphyrin biosynthetic pathway is divided into four main parts: (1) the formation of 5-aminolevulinic acid

(ALA), (2) the formation of protoporphyrin IX (Proto IX) from eight molecules of ALA, (3) the magnesium porphyrin (Mg-porphyrin) branch to chlorophyll, and (4) the heme-synthesizing branch (Papenbrock and Grimm, 2001; Tanaka and Tanaka, 2007) (Fig. 1).

The biosynthesis of porphyrin is tightly regulated at several levels to coordinate apoprotein synthesis and to avoid the accumulation of porphyrin intermediates (Papenbrock and Grimm, 2001). Plants suffer severe photodynamic damage if these control mechanisms are circumvented. All chlorophyll precursors are potent photosensitizers, so their accumulations will interact with molecular oxygen in the presence of light to produce ROS, such as singlet oxygen and hydrogen peroxide, which cause damage to proteins, lipids, carbohydrates, pigments, and DNA and ultimately result in cell death (Kim *et al.,* 2014; Phung and Jung, 2014 & 2015). Porphyrin intermediate biosynthesis may provide signals to control expression of nuclear genes in response to metabolic activity in chloroplasts (Chi *et al*., 2013). Several studies have been performed to investigate the impact of water stress on photosynthesis (Galmés *et al*., 2007; Massacci *et al*., 2008), however, there were little known about porphyrin biosynthesis in response to drought stress. In our study, important products, key enzymes and related genes in porphyrin biosynthetic pathway were investigated after withholding irrigation to reveal the metabolic regulation of the porphyrin biosynthetic pathway under drought condition.

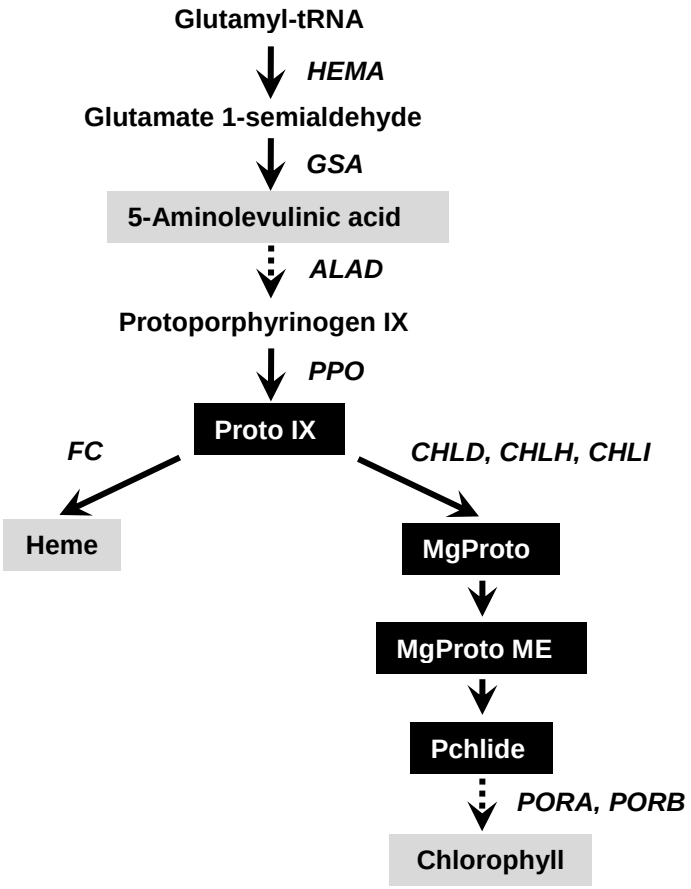

Fig. 1. The tetrapyrrole pathway in plants showing intermediates and genes analyzed in this study

Note: Intermediates quantified in this study are highlighted. Intermediates: Proto IX, MgProto, MgProto ME, Pchlide. Genes and enzymes that correspond to the gene names: HEMA, GSA, ALAD, PPO, FC, CHLD, CHLH, CHLI, PORA, PORB

2. MATERIALS AND METHODS

2.1. Materials

Rice seedlings (*Oryza sativa* cv. Dongjin fromKorea) were used in this study. They were grown in growth chamber maintained at day/night temperatures of 28°C/25°C under a 14-h-light/10-h-dark cycle (7:00 AM-9:00 PM) with 200 mmol m^{-2} s^{-1} photosynthetic photon flux density.

2.2. Methods

- Drought treatment: Three-week-old rice seedlings were exposed to drought by withholding water for 60 h. Youngest, fully expanded leaf tissues were sampled at 36 h (9:00 AM), 48 h (9:00 PM), 60 h (9:00 AM) after drought treatment. Control plants with sufficient water supply were harvested at the same time as the drought-treated plants for 36 h as previously described (Phung Thi Thu Ha, 2014).

- Determination of porphyrin: For measurement of porphyrin content, plant tissue was extracted then separated and measured by HPLC following the method of Lermontova and Grimm (2006).

- Determination of heme: Heme was extracted and separated by HPLC as described previously (Schneegurt and Beale, 1986).

- *ALA-synthesizing capacity*: ALA-synthesizing capacity was measured using spectrophotometer as described by Papenbrock *et al.* (1999).

- Assays for enzyme activities of tetrapyrrole biosynthesis: The PPO activity was determined using the method of Lermontova and Grimm (2000). Mg-chelatase was assayed as described by Lee *et al.* (1992). Fe-chelatase activity was measured using the protocol from Papenbrock *et al.* (1999).

- *RNA extraction and RT-PCR:* Total RNA was prepared from leaf tissues using TRIZOL Reagent (Invitrogen), and 5 mg of RNA from each sample was used for the reverse transcription reaction (SuperScript III First-Strand Synthesis System; Invitrogen). Subsequently, 50 ng of cDNA was used for RT-PCR analysis. The specific primers were designed based on gene bank database as described previously by Phung *et al.* (2011). Actin was used as the internal control.

Data were analyzed by Microsoft Excel. The data represent the mean ± SE of six replicates from two independent experiments.

3. RESULTS AND DISCUSSION

3.1. Effect of drought on porphyrin intermediates and end products

Rice seedlings were drought treated by withholding irrigation for 60 h. The drought symptom expressed as leaves rolling after 36, 48 and 60 h of treatment. At 60 h after drought treatment, the dehydration symptom was more severe; the leaves lost more than 60% of water content (Fig. 2) as reported in previous studies (Phung Thi Thu Ha, 2014).

To study the effect of drought stress on porphyrin metabolic flux, we first monitored the concentration of Proto XI, the common precursor of both heme and chlorophyll branches, and three intermediates of chlorophyll branch (MgProto, MgProto ME, and Pchlide) before and after withholding water for 36, 48 and 60 h. The result showed that content of all porphyrin intermediates greatly decreased after drought stress in leaves of the rice seedlings. These levels significantly decreased at 36 h of drought treatment although treated plants did not yet exhibit any drought symptom. At 60 h after withholding irrigation, the levels remained in small amount, even Proto IX almost disappeared from the leaves of treated plants (Fig. 3). The decrease of the porphyrin intermediates was directly proportional to the accumulation of H_2O_2 and malondialdehyde (Fig. 3).

Among the end products of porphyrin pathway, chlorophyll is an important pigment in photosynthetic system of photosynthetic organisms and heme is an essential molecule that is responsible for crucial biological activities including oxygen metabolism and transfer, electron transfer and secondary metabolism (Tanaka and Tanaka, 2007). In porphyrin biosynthetic pathway, drought stress

did not much affect chlorophyll content, whereas heme content slightly reduced in the leaves of treated seedlings (Fig. 3). Taken together with the reduction of porphyrin intermediates (Fig. 3), it indicated that drought stress affected porphyrin intermediates more than the end products. The similar tendency was found in plants responding to chilling, heat and salt stress in previous publication. Phung and Jung (2015) reported that the content of porphyrin intermedieates (Proto IX, MgProto, MgProto ME and Pchlide) drastically declined whereas chlorophyll content slightly decreased only in rice plants under chilling and heat stress. And salt stress led to the reduction in content of Proto IX and MgProto ME but did not affect chlorophyll content (Yun *et al.*, 2012). The excess of porphyrin photosensitizers also caused the accumulation of ROS which damage plant cells (Kim *et al.*, 2014; Phung and Jung, 2014 & 2015), so the degradation dynamics of these photosensitizing porphyrin may lead to reduced ROS production and altered redox state of plastids (Phung *et at.,* 2011) in order to protect plants from drought damage. It may be an additional protective mechanism of plants in response to stress.

3.2. Effect of drought on the expression of nuclear-encoded photosynthetic genes

Retrograde signaling is a process in which plant organelles emit signals that regulate the expression of nuclear genes. The chlorophyll intermediate MgProto as one of such signal acts as a negative regulator of photosynthetic gene expression (Nott *et al.*, 2006). The expression levels of nucleus-encoded photosynthetic genes (*Lhcb1*, *Lhcb6* and *RcbS*) were determined to evaluate their relationship with MgProto under drought stress. The results showed that transcription levels of *Lhcb1* and *Lhcb6*, the genes encoding Lhcb protein of Photosystem II, were down-regulated under water deficit with an earlier decline of *Lhcb6* than *Lhcb1*. The expression of *rcbS* gene encoding the small subunit of Rubisco, also drastically reduced after 48 h of withholding water (Fig. 4). The reduction of MgProto content together with a down-regulation of *Lhcb1*, *Lhcb6* and *RbcS* genes was aslo found in oxyfluorfen- and ALA- treated plants after two days (Phung and Jung, 2014). By contrast, MgProto accumulation led to a down-regulation of *Lhcb* and other nucleus-encoded photosynthetic genes under norflurazon treatment (Strand *et al.*, 2003). Our data indicated that down-regulation of nuclear-encoded photosynthetic genes not due to the accumulation of MgProto but also plant cell damage and accumulation of ROS in rice seedling after stopping irrigation and it also lead to reduced efficiency of photosystem II (Fig. 3 & 4).

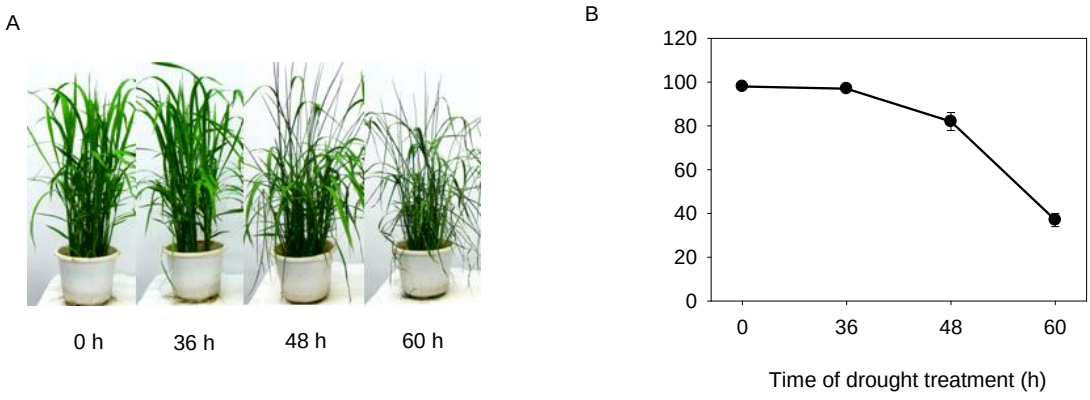

Fig. 2. (A) Phenotypes of rice seedlings and (B) relative water content (RWC) of leaves before and after water withholding for 36 h, 48 h and 60 h

Source: Phung Thi Thu Ha, 2014

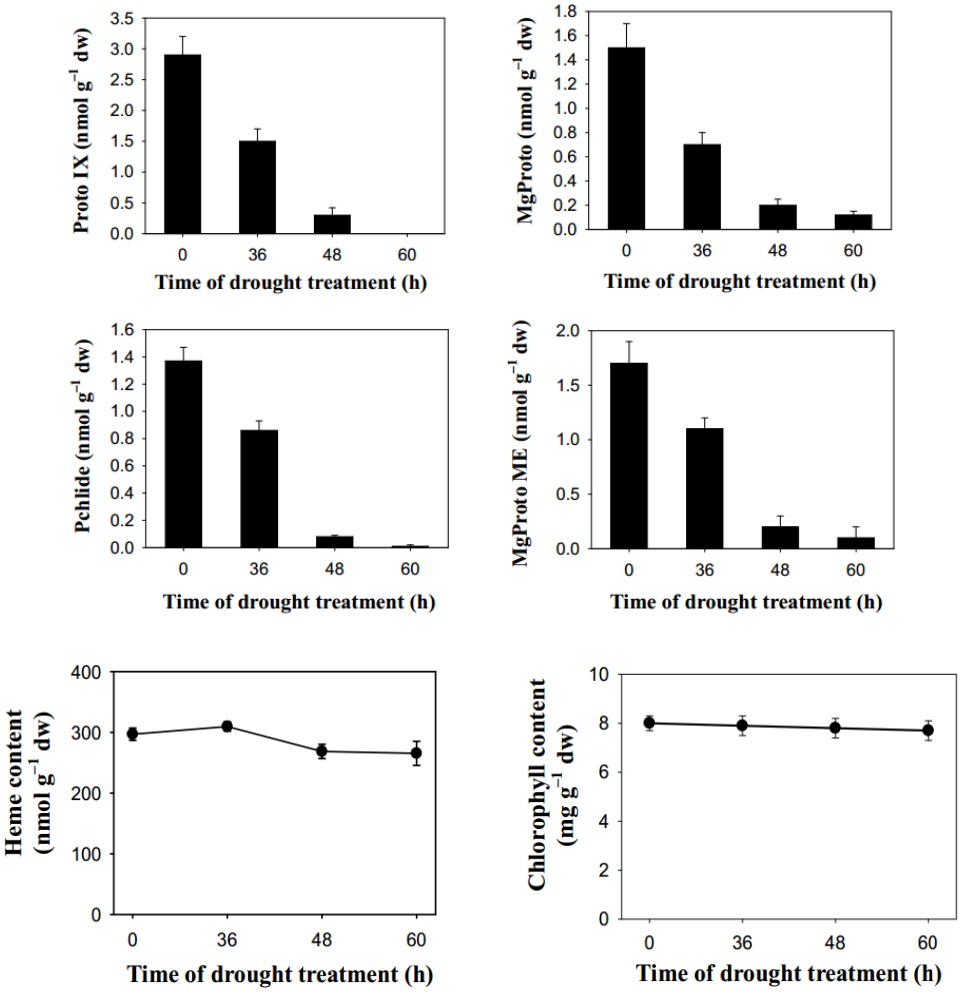

Fig. 3. Effect of drought on tetraphyrrole metabolites flux in rice seedling

Note: The plants were subjected to the same treatments as in Fig. 2. Treatment notations are the same as in Fig. 2. Values are means ± SE of six replicates from two independent experiments

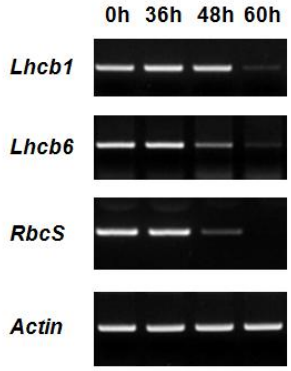

Fig. 4. The expression of nuclear-encoded photosynthetic genes in drought-stressed leaves

Note: Expression analysis of nuclear-encoded photosynthetic genes by RT-PCR. Total RNAs were purified from plants and reverse-transcribed. The resultant cDNAs were used as templates for RT-PCR using Actin as an internal control. The plants were subjected to the same treatments as in Figure 2

3.3. Effect of drought on enzyme activities of porphyrin biosynthetic pathway and gene expression

In the subsequent study, the activity of some key enzymes in porphyrin biosynthetic pathway was examined. The first step is the corporation of some enzymes in the early of porphyrin pathway scheme to produce ALA, the important precursor of this pathway. ALA synthesizing capacity decreased in rice seedlings in response to drought stress from 48 h after withholding water (Fig. 5). The data correlated with the expression of three corresponding genes *HEMA1*, *GSA* and *ALAD* (Fig. 6). Jain *et al.* (2013) also reported that ALAD activity reduced in leaves of etiolated maize seedlings under water deficit induced by PEG-6000 which led to reduction of ALA content, the first important precursor of porphyrin pathway.

The second enzyme monitored was PPO activity. PPO enzyme catalyzes the formation of Proto IX from Protogen IX. PPO activity declined in rice plants after 36 h of treatment (Fig. 5). It correlated with the reduction of *PPO* gene expression in response to water deficit (Fig. 6).

Mg-chelatase and Fe-Chelatase are two first enzymes of chlorophyll and heme branch, respectively. Both use Proto IX produced by PPO enzyme as a substrate. From Proto IX, Fe-chelatase converts to heme and Mg-chelatase to produce chlorophyll, the end products of these branches. Their activities decreased in treated plants in response to drought. However, Mg-chelatase activity exhibited more drastic decline than Fe-chelatase activity as well as PPO activity and ALA synthesizing capacity (Fig. 5). The expression of chlorophyll branch genes (*CHLH*, *CHLI*, *CHLD*, *PORA*, *PORB*) and heme branch gene (*FC2*) were also down-regulated in rice plants after drought treatment (Fig. 6). Those data indicated that drought stress affected chlorophyll branch more than heme branch. This might relate to the role of heme in detoxification system.

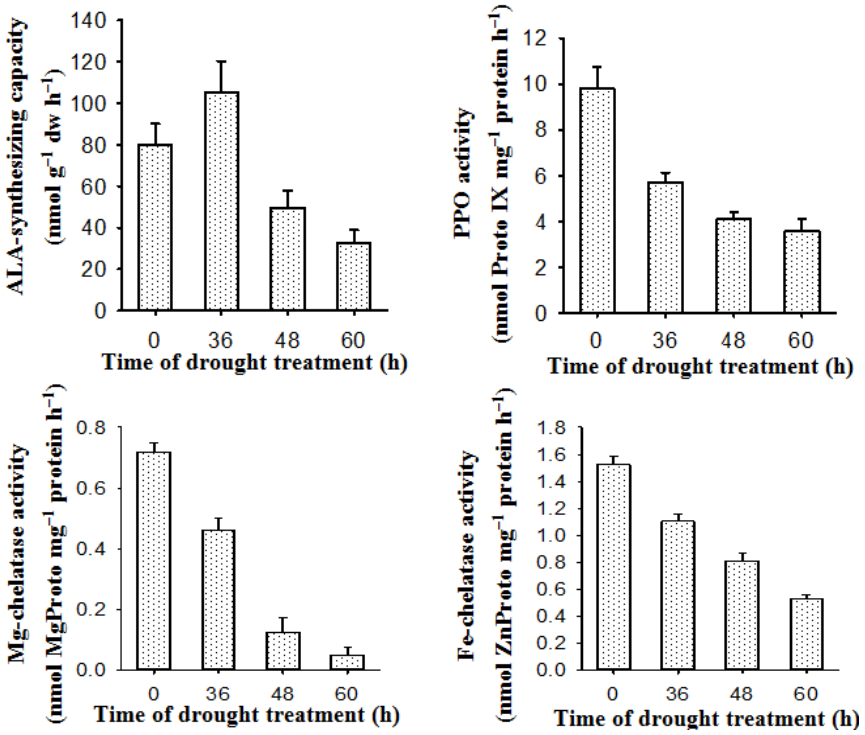

Fig. 5. Effect of drought on porphyrin synthesizing enzyme activity in leaves of rice seedling

Note: The plants were subjected to the same treatments as in Fig. 2. Treatment notations are the same as in Fig. 2. Values are means ± SE of six replicates from two independent experiments

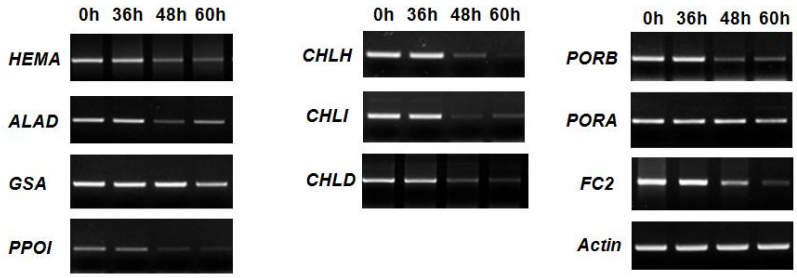

Fig. 6. Drought-induced changes in expression of genes encoding the porphyrin pathway enzymes

Note: Total RNAs were purified from plants and reverse-transcribed. The resultant cDNAs were used as templates for RT-PCR using Actin as an internal control. The plants were subjected to the same treatments as in Figure 2. Although activity of Mg-chelatase, expression of chlorophyll branch genes and chlorophyll intermediates decreased under drought, chlorophyll content slightly decreased only (Fig 3, 5, 6). It is in agreement with Jain et al. *(2013) that water deficit affects chlorophyll formation rather than its degradation. Down-regulation of the porphyrin biosynthesis genes and enzyme activity have also been shown in chilling-stressed seedlins (Mohanty* et al.*, 2006) and in oxyfluorfen - and ALA- treated rice plants (Phung and Jung, 2014).*

Our data indicated a sensitivity of porphyrin pathway to drought stress. It also demonstrated a tight control of porphyrin biosynthesis in order to prevent the accumulation of toxic metabolic intermediates by down-regulation of their biosynthesis under drought condition.

4. CONCLUSION

In this study, rice plants which were drought treated by withholding irrigation led to the down-regulation of porphyrin metabolite flux (including porphyrin intermediates and the end products, the activity of key enzymes and the expression of porphyrin biosynthetic genes) in order to prevent the accumulation of harmful singlet oxygen generating porphyrins. It also caused the reduced expression of nuclear-encoded photosynthesis genes (*Lhcb1*, *Lhcb6* and *RbcS*) as a result of cellular damage by ROS accumulation.

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