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Clade Ib basic helix-loop-helix transcription factor, bHLH101, acts as a regulatory component in photo-oxidative stress responses

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ABSTRACT

The accumulation of reactive oxygen species (ROS) leads to oxidative damage; however, ROS also acts as signaling molecules. We previously demonstrated that the inducible silencing of thylakoid membrane-bound ascorbate peroxidase *Arabidopsis* plants (IS-tAPX) accumulated H_2O_2 in their chloroplasts, resulting in the clarification of ROS-responsive genes. In IS-tAPX plants, the transcript levels of the basic helix-loop-helix (bHLH) transcription factor *bHLH101,* which belongs to clade Ib bHLH, were down-regulated. In order to investigate the participation of bHLH101 in chloroplastic H_2O_2-mediated signaling, we isolated dominant negative expression mutants of *bHLH101* (*DN-bHLH101*). *DN-bHLH101* plants showed a significant phenotype that was sensitive to a methylviologen treatment, even under iron-sufficient conditions. Furthermore, the knock out mutant of *bHLH101* showed a photo-oxidative sensitive phenotype, indicating that other clade Ib bHLHs do not compensate for the function of bHLH101. Thus, bHLH101 appears to act as a regulatory component in photo-oxidative stress responses. We also found that ferric chelate reductase activity was stronger in IS-tAPX plants than in control plants, suggesting that there is a close relationship between iron metabolism and oxidative stress responses.

1. Introduction

Iron is a metal element that is essential for many metabolic processes including photosynthesis, respiration, and DNA synthesis in plants. Although iron is abundant in soil, it is mainly present in its oxidized form, Fe^{3+}, the solubility of which is low. Furthermore, in alkaline and calcareous soils, the levels of bio-available iron are generally too low and iron uptake is strongly inhibited. These factors have marked consequences on plant productivity in calcareous soils. Plants have developed efficient iron-uptake systems to cope with iron deficiencies. In *Arabidopsis*, the central transcriptional regulator in iron acquisition is Fe-regulated (Fer)-like iron-deficiency-induced transcription factor (FIT), a basic helix-loop-helix (bHLH) transcription factor orthologous to the tomato (*Solanum lycopersicum*) FER protein [1–4]. The *FIT*-null mutation, *fit-1*, is lethal at the seedling stage without an extra iron supply and FIT regulates the expression of iron-deficiency-inducible genes, indicating that it is essential for normal growth and development under iron-sufficient conditions [2]. Moreover, FIT forms heterodimers with one of the four subgroup Ib bHLH proteins, bHLH38, bLHL39, bHLH100, and bHLH101, the expressions of which is induced under low iron levels [5,6]. The bHLH transcription factor POPEYE (PYE) has been identified as a second transcriptional regulator of iron acquisition. The *pye-1* mutant has shown sensitivity to iron deficiencies, and PYE regulates the expression of the iron-transport-related genes, *NAS4*, *FRO3*, and *ZIF1* [7].

Since excess iron generates cytotoxic hydroxyl radicals via the Fenton reaction, the maintenance of proper cellular levels of iron is essential for normal growth and development in plants. Iron is toxic for

Abbreviations: APX, ascorbate peroxidase; AsA, ascorbic acid; bHLH, basic helix-loop-helix; BPDS, bathophenanthroline disulfonate; FCR, Ferric chelate reductase; Fer, Fe-regulated; FIT, Fer-like iron-deficiency-induced transcription factor; HL, high light; ML, moderate light; MS, Murashige & Skoog; MV, methylviologen; NL, normal light; PYE, POPEYE; q-PCR, quantitative real-time PCR; ROS, reactive oxygen species; RTS, responsive to tAPX silencing; SRDX, SUPERMAN repression domain X; tAPX, thylakoid membrane-bound ascorbate peroxidase

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the photosystem electron transfer components, leading to the generation of reactive oxygen species (ROS) and peroxidation chain reactions in membrane lipids. Most organisms deploy an array of mechanisms to control cellular iron homeostasis and repair damaged cellular structures [8]. Chlorosis is the first visible symptom in iron-deficient plants. The activities of various enzymes, which require iron as an essential component of heme- or iron-sulfur complexes, were previously shown to be reduced under iron-deficient conditions [9]. A decrease in iron levels disrupts the metabolic balance of a cell, resulting in the over-accumulation of ROS, which trigger cell death. The chlorotic phenotype under iron-deficient conditions was recovered by supplying antioxidants [9]. Thus, the disruption of iron homeostasis in plant cells induces oxidative damage that arises from ROS. These findings suggest that the redox regulation of the antioxidant system is closely related to iron homeostasis [10].

ROS plays a dual role in plant cells as toxic compounds and signaling molecules. A previous study suggested that there are source- and kind-specific pathways for ROS signaling in plant cells [11]. In order to clarify the signaling function of H_2O_2 derived from chloroplasts, we created a system for producing H_2O_2 in *Arabidopsis* chloroplasts by chemically inducing the silencing of thylakoid membrane-bound ascorbate peroxidase (tAPX) [12]. In the assay, the significant accumulation of salicylic acid and the induction of defense genes were observed in tAPX-silenced plants (IS-tAPX) without any other stress application. In addition, this assay identified a set of putative chloroplastic H_2O_2-responsive genes, named *responsive to tAPX silencing* (*RTS*). In order to clarify the molecular mechanisms underlying chloroplastic H_2O_2-mediated signaling, we isolated some oxidative stress-sensitive and -insensitive mutants from *Arabidopsis* lines in which RTS genes were knocked out [13–15]. In addition to these mutants, we newly isolated an oxidative stress sensitive mutant that exhibited the dominant negative expression of *bHLH101*, which was involved in iron-deficient responses. These mutants showed greater sensitivity to oxidative stress than wild-type plants, regardless of whether iron was supplied, suggesting that bHLH101 is related to oxidative stress tolerance. Furthermore, we found that H_2O_2 derived from chloroplasts functions as a positive signal factor for iron uptake in IS-tAPX plants. These results suggest a relationship between chloroplastic H_2O_2 and iron uptake in plant responses to oxidative stress.

2. Materials and methods

2.1. Plant materials and growth conditions

Wild-type seeds of *Arabidopsis* (*Arabidopsis thaliana*) ecotype Columbia-0 (Col-0) and mutant seeds were sown on 1/2 Murashige & Skoog (MS) medium containing 1% sucrose and 5–100 μM Fe at 23 °C under a continuous light source (100 μmol photons m^{-2} s^{-1}). All seeds were placed at 4 °C in the dark for 2 days to break dormancy.

The generation and characterization of IS-tAPX and IS-GUS plants was described previously [12]. In order to generate the dominant negative expression of *bHLH101* plants, the coding regions of bHLH101 were amplified and cloned into the *Sma* I site of the p35SSRDXG vector [16]. The constructed vector was transferred into the pBCKH plant expression vector [16] using the Gateway system (Invitrogen). The plant expression vector pRI101-AN (Takara, Shiga, Japan) was used for the generation of bHLH101-overexpression *Arabidopsis* plants. The cDNA coding sequences of bHLH101 were amplified with primers containing *Nde* I and *Eco* RI at the 5′ and 3′ ends of the bHLH101 fragment and were ligated into pRI101-AN between the 35S CaMV promoter and nopaline synthase polyadenylation signal. These constructs were transformed into *Agrobacterium tumefaciens* strain EHA105 and transformed to *Arabidopsis* by the floral-dip method. Transformed seeds were selected on MS plates containing 50 μg mL^{-1} hygromycin B or kanamycin. The single insertion homozygote lines from T_3 generation were used in experiments. The T-DNA insertion line of bHLH101 (*KO-bhlh101*; SALK_011245) was obtained from the Arabidopsis Biological Resource Center.

2.2. Quantitative real-time PCR (q-PCR)

q-PCR experiments were performed with a LightCycler96 System using the FastStart Universal SYBR Green Master (ROX) (Roche, Basal, Switzerland) according to Noshi et al. [15]. Total RNA was isolated from the leaves or roots of *Arabidopsis* plants using Sepasol-RNA I (Nacalai Tesque, Kyoto, Japan). Total RNA was treated with DNaseI (Thermo Fisher Scientific, Waltham, MA). First-strand cDNA was synthesized using reverse transcriptase (ReverTra Ace, Toyobo, Osaka, Japan) with an oligo dT_{20} primer according to the manufacturer's instructions. Primer pairs for q-PCR were designed using PRIMER EXPRESS software (Applied Biosystems, CA, USA). Gene-specific primers were selected such that the resulting PCR product had an approximately equal size of 100 bp. *ActinII* was used as an internal standard in all experiments. At least three experimental replicates were used for one biological replicate.

2.3. Semi-quantitative RT-PCR experiments

A semi-quantitative RT-PCR analysis was performed according to Maruta et al. [12]. Semi-quantitative RT-PCR experiments were repeated at least three times with cDNA prepared from three batches of plant leaves. *Actin8* was used as control.

2.4. Measurement of chlorophyll, anthocyanin, and Fv/Fm

Chlorophyll was extracted from *Arabidopsis* leaves using 80% (w/v) acetone. The amount of chlorophyll was assessed according to Arnon [17]. The measurement of anthocyanin was performed according to Maruta et al. [14]. *Arabidopsis* leaves (50 – 100 mg) were homogenized in 1 ml of extraction buffer [18% (v/v) 1-propanol and 1% (v/v) HCl] and agitated for 1 h. The homogenate was boiled in a water bath for 30 min and then incubated at 23 °C in the dark for 10 h. The homogenate was centrifuged at 10,000 x *g* for 10 min. The absorbance (A535-A650) of anthocyanin in the supernatant was measured with a Shimadzu UV-1700 spectrophotometer. The amount of anthocyanin was calculated per g FW. Chlorophyll fluorescence was measured according to Noshi et al. [18]. *Arabidopsis* plants were placed in the dark for 20 min in order to assess F_0 and F_m. Fluorescence parameters were calculated as follows: $F_v/F_m = (F_m\text{-}F_0)/F_m$. Chlorophyll fluorescence in *Arabidopsis* leaves was measured at 23 °C with a Closed FluorCam 800 M F (Photon Systems Instruments, Brno, Czech Republic). At least 20 independent wild-type and mutant plants were used in this assay.

2.5. Enzyme assay

APX activity was measured according to Yoshimura et al. [19]. *Arabidopsis* leaves (0.3 g) were ground to a fine powder in liquid N_2 and then homogenized in 1 ml of 100 mM potassium phosphate buffer (pH 7.6), 20% (w/v) sorbitol, 1 mM EDTA, 5 mM Ascorbic acid (AsA), and 2% (w/v) polyvinylpyrrolidone. The homogenate was centrifuged 12,000 x *g* at 4 °C for 10 min. The supernatant, which contains the activities of stromal APX and cytosolic APX isoenzymes was used to measure APX activity. The activities of APX isoenzymes were measured as a decrease in absorbance at 290 nm (e = 2.8 mM^{-1} cm^{-1}) due to AsA oxidation.

Ferric chelate reductase (FCR) was measured using the bathophenanthroline disulfonate (BPDS) assay as described by Lei et al. [20] with some modifications. Whole plants (12–15 plants) were excised and then rinsed with 300 μM $CaSO_4$ solution and demineralized water three times. The rinsed roots were soaked in assay solution (5 mM MES-NaOH pH 5.5, 300 μM $CaSO_4$, 300 μM BPDS, and 100 μM Fe^{3+}- EDTA). Reactions were incubated at 25 °C for 40 min in the dark. The content of