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Ferulic acid 5-hydroxylase 1 is essential for expression of anthocyanin biosynthesis-associated genes and anthocyanin accumulation under photooxidative stress in *Arabidopsis*

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ABSTRACT

Anthocyanins are important for preventing photoinhibition and photodamage. By comprehensive reverse genetic analysis of chloroplast-produced H_2O_2-responsive genes, we isolated here an anthocyanin-deficient mutant under photooxidative stress, which lacked ferulate 5-hydroxylase 1 (FAH1) involved in the phenylpropanoid pathway. Interestingly, the expression of anthocyanin biosynthesis-associated genes was also inhibited in this mutant. These findings suggest that FAH1 is essential for expression of anthocyanin biosynthesis-associated genes and anthocyanin accumulation under photooxidative stress in *Arabidopsis*. Furthermore, we found that estrogen-inducible silencing of thylakoid membrane-bound ascorbate peroxidase, which is a major H_2O_2-scavenging enzyme in chloroplasts, enhances the expression of *FAH1* and anthocyanin biosynthesis-associated genes and accumulation of anthocyanin without any application of stress. Thus, it is likely that chloroplastic H_2O_2 activates *FAH1* expression to induce anthocyanin accumulation for protecting cells from photooxidative stress.

1. Introduction

Reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide radical, singlet oxygen, and hydroxyl radical act as signaling molecules associated with responses to abiotic and biotic stress in living organisms including animals, plants, and microbes [1]. ROS are mainly produced during central metabolic functions such as photosynthesis, respiration, and photorespiration. Therefore, chloroplasts, mitochondria, and peroxisomes are main sources of ROS in plant cells [2], though plants contain ROS-producing enzymes such as NADPH oxidase in apoplast [3,4]. It has been suggested that there are source- and kind-specific pathways for ROS signaling in plant cells [5]. The synergistic and antagonistic interactions of multiple signaling pathways are thought to be important for the fine-tuning of responses to abiotic and biotic stress.

In plant cells, chloroplasts are one of the most significant sources of ROS [2,6,7]. The ROS generated in chloroplasts act as a retrograde signal to the nucleus for regulating plant responses to biotic and abiotic stress [2,8,9]. Thylakoid membrane-bound ascorbate peroxidase (tAPX) is a major H_2O_2-scavenging enzyme in chloroplasts [6,10,11]. To clarify the signaling function of chloroplastic H_2O_2, we created a novel system for producing H_2O_2 in *Arabidopsis* chloroplasts by estrogen-inducible silencing of tAPX [12]. Microarray analysis revealed that the transient reduction of tAPX affects the expression of 774 genes, which are named *Responsive to tAPX Silencing* (RTS). In addition to the functional classification of RTS genes, physiological analyses using tAPX-silenced plants revealed that chloroplastic H_2O_2 negatively regulates cold acclimation, but positively regulates the response to salicylic acid, a phytohormone [12,13]. Chloroplastic H_2O_2 was also involved in the response to photooxidative stress, such as high light (HL) exposure [12]. Interestingly, it seems that there are positive and negative roles for chloroplastic H_2O_2 in photooxidative stress response [10,12]. Although the levels of H_2O_2 in chloroplasts were enhanced in knockout and knockdown mutants of tAPX, the expression of

Abbreviations: 4CL, 4-coumarate:CoA ligase; APX, ascorbate peroxidase; COMT, caffeic acid/5-hydroxyferulic acid O-methyltransferase; CHI, chalcone isomerase; CHS, chalcone synthase; C4H, cinnamate 4-hydroxylase; CAD, cinnamyl alcohol dehydrogenase; DFR, dihydroflavonol 4-reductase; FAH1, ferulate 5-hydroxylase 1; F3H, flavanone 3-hydroxylase; F3′H, flavonoid 3′-hydroxylase; HCT, hydroxycinnamoyl-CoA shikimate:quinate hydroxycinnamoyl-transferase; LDOX, lencoanthocyanidin dioxygenase; MV, methylviologen; PAL, phenylalanine ammonia-lyase; ROS, reactive oxygen species; RTS, responsive to tAPX silencing.

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ROS- and HL-responsive genes such as cytosolic APX and heat shock factor A2 was inhibited in these mutants during HL [10,12], suggesting a negative effect of chloroplastic H_2O_2 on photooxidative stress response. In contrast, the positive effect is poorly understood.

Plants have multiple defense systems against photooxidative stress. Thus, they highly accumulate ascorbate and glutathione as antioxidants and have a diverse set of antioxidative enzymes including APXs, superoxide dismutases, and glutathione peroxidases [2,7,10,14]. In addition, anthocyanins are important for preventing photoinhibition and photodamage through the absorption of excessive solar radiation that would otherwise be absorbed by chloroplast pigments [15,16]. In fact, plants highly accumulate anthocyanins under HL [17]. As shown in Fig. 1, anthocyanins as well as flavonols, the main flavonoids in *Arabidopsis*, are synthesized from malonyl-CoA and phenylalanine, an intermediate of the phenylpropanoid pathway [18,19]. The molecular mechanisms behind the biosynthesis of phenylpropanoid and anthocyanins are well characterized. However, the involvement of ROS and cellular redox states in the regulation of these pathways is still under discussion.

To clarify the molecular mechanism and physiological function of chloroplastic H_2O_2-mediated signaling, we have been trying to isolate oxidative stress-sensitive and -insensitive mutants from lines of *Arabidopsis* in which RTS genes are knocked out [20]. Here, we found an anthocyanin-deficient mutant under photooxidative stress, which lacked ferulate 5-hydroxylase 1 (FAH1) involved in the phenylpropanoid pathway [19]. The expression of anthocyanin biosynthesis-associated genes was also inhibited in this mutant. Furthermore, silencing of tAPX enhanced the expression of *FAH1* and accumulation of anthocyanin without any application of stress. These findings suggest that FAH1 is essential for expression of anthocyanin biosynthesis-associated genes and anthocyanin accumulation under photooxidative stress, and provide a new insight into the positive role of chloroplastic H_2O_2 in photooxidative stress response.

2. Materials and methods

2.1. Plant materials and growth conditions

Arabidopsis thaliana (Col-0) was used as the wild-type plants. T-DNA insertion lines of RTS genes including *fah1-101* (SAIL_80_C01) in the Col-0 background were obtained from the Arabidopsis Biological Resource Center (ABRC). The mutant lines are listed in Supplementary Table S1. Other *fah1* alleles, *fah1-2* and *fah1-7*, and a *reduced epidermal fluorescence* (*ref*) mutant, *ref1-4*, were also obtained from ABRC. To isolate oxidative stress-sensitive and -insensitive mutants, 20 seeds of respective T-DNA insertion lines and wild-type plants were surface sterilized, imbibed, and vernalized for 2 days (4 °C, dark) in tubes. The seeds of the T-DNA insertion lines and wild type were separately sown on half-strength Murashige and Skoog (MS) medium containing 1% sucrose with 0.1 μM methylviologen (MV), a ROS-producing agent in chloroplasts and mitochondria. Plants were grown under normal light (NL, 100 μmol photons m^{-2} s^{-1}) at 25 °C for 2 weeks, and phenotypes of the T-DNA insertion lines were carefully compared to those of wild-type plants during seedling development.

The wild-type and *fah1-101* plants were also grown on the MS medium without MV under moderate light (ML) at 400 μmol photons m^{-2} s^{-1} to induce photooxidative stress. For a short-term application of photooxidative stress, 2-week-old wild-type and/or *fah1-101* plants grown on the MS medium under NL conditions were exposed to high light (HL) at 1000 μmol photons m^{-2} s^{-1} or sprayed with 25 μM MV. In any case, we used continuous light conditions for plant growth and stress application to abolish light/dark effects on the production of chloroplastic H_2O_2.

The generation and properties of the IS-tAPX-19 (for tAPX silencing) and IS-GUS-2 (for control) plants were described previously by Maruta et al. [12]. The IS-tAPX-19 plants was introduced an RNAi construct having an inverted repeat corresponding to a 530-bp fragment of the 3′-terminal region of *tAPX* under control of an estrogen-inducible promoter. These transgenic plants were grown on the MS medium under continuous NL conditions for 1 week, and then transferred to soil. Seventeen-day-old IS-tAPX-19 and IS-GUS-2 plants were sprayed with 100 μM estrogen to induce RNAi for tAPX silencing.

2.2. Measurement of anthocyanin, chlorophyll, and malondialdehyde

For the extraction of anthocyanin, *Arabidopsis* leaves (50–100 mg) were homogenized in 1 ml of extraction buffer [18% (v/v) 1-propanol and 1% (v/v) HCl] and shaken for 1 h. The homogenate was boiled in a water bath for 30 min and incubated for 8–10 h at 23 °C in darkness. The supernatant was obtained by centrifugation at 10,000 × *g* for 10 min. The absorbance (A_{535}–A_{650}) of anthocyanin in the supernatant was quantified spectrophotometrically (PharmaSpec UV-1700; Shimadzu); the amount of anthocyanin was calculated per g FW. Chlorophyll was extracted at 4 °C from 0.2 g of seedlings using acetone, and the amount was determined according to Arnon [21].

The thiobarbituric acid test was used to determine malondialdehyde (MDA) as an end-product of lipid peroxidation [22]. The MDA contents were estimated by measuring absorbance from A_{532} to A_{600} and using a molar absorption coefficient of 1.56×10^5 [23].

2.3. Leaf area determination

Leaf area measurements were performed as described by Schulz et al. [24]. Two-week-old seedlings were photographed. Total rosette surface area (hereafter called leaf area) was measured and analyzed using ImageJ as described by Schindelin et al. [25]. Data was processed using Microsoft Excel and statistical analysis was performed with a *t*-test.

2.4. Semi-quantitative RT-PCR experiments

Total RNA was isolated from the leaves of *A. thaliana* ecotype Columbia (Col-0) according to Matuta et al. [26]. First strand cDNA was synthesized using ReverTra Ace reverse transcriptase (TOYOBO, Osaka, Japan) with an oligo dT primer. The semi-quantitative RT-PCR experiments were performed according to Ogawa et al. [27]. Primer sequences are as follows; FAH1-F (5′-CGGTCGGTCTCTTGTAACGTTG-3′), FAH1-R (5′-CAACTCGTCTGTCAAGTCCAACGA-3′), Actin8-F (5′-GAGATCCACATCTGCTGG-3′), and Actin8-R (5′-GCTGAGAGATTCAGGTGCCC-3′).

2.5. Quantitative Real-Time PCR experiments

Quantitative Real-Time PCR (q-PCR) experiments were performed according to Nishizawa et al. [28]. Primer pairs for q-PCR were designed using PRIMER EXPRESS software (Applied Biosystems); primer sequences as well as accession numbers of genes are shown in Supplementary Table S2. Gene-specific primers were chosen such that the resulting PCR product had an approximately equal size of 100 bp. q-PCR was performed with an Applied Biosystems 7300 Real Time PCR System, using the SYBR Premix Ex Taq (Takara). *Actin2* mRNA was used as an internal standard in all experiments. We confirmed that the expression of *Actin2* is not changed in our experimental conditions by semi-quantitative RT-PCR (data