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Identification and characterization of the BAHD acyltransferase malonyl CoA: Anthocyanidin 5-*O*-glucoside-6″-*O*-malonyltransferase (At5MAT) in *Arabidopsis thaliana*

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Abstract **The major anthocyanin in *A. thaliana* is a cyanidin derivative modified by glycosylation as well as by the addition of three acyl moieties: malonyl, *p*-coumaroyl, and sinapoyl. We have isolated a member of the BAHD acyltransferase family which catalyzes this malonylation reaction by combining a reverse genetics approach with biochemical genomics. A mutant line containing a T-DNA insertion in *At3g29590*, the gene encoding the malonylating enzyme, is incapable of producing malonylated anthocyanins. Transgenic plants harboring an RNAi silencing cassette for *At3g29590* demonstrate a positive correlation between reduction in the *At3g29590* gene transcript and the decrease of malonylated anthocyanins. Transcript levels for both *At3g29590* and the epistatic gene *At4g14090*, encoding 5-*O*-anthocyanin glucosyltransferase, increase in several plant lines as they accumulate anthocyanin pigments. Investigation of the heterologously expressed and purified malonylating enzyme showed that the activity is specific for malonyl-CoA and for anthocyanins with 5-*O*-glucosylation. The malonyl transfer itself occurs only to the 5-*O*-glucoside function, and not to any of the other sugar moieties present in *A. thaliana* anthocyanins. Hence, both *in vivo* and *in vitro* results define the activity of the At3g29590-encoded enzyme as an anthocyanin 5-*O*-glucoside-6″-*O*-malonyltransferase (At5MAT).**

Keywords: Anthocyanin; BAHD; Acyltransferase; Malonyl coenzyme A; *Arabidopsis thaliana*

1. Introduction

Anthocyanins are a large class of glycosylated flavonoid compounds responsible for nearly all the purple, blue and pink pigmentation of angiosperm leaves and flowers and much of the red and orange as well. To date, more than 539 different anthocyanin structures have been reported [1]. The large chemical diversity found in this class of plant secondary metabolites is achieved through chemical modifications of the core anthocyanidin structure, a 2-(4′-hydroxyphenyl)-3,5,7-trihydroxybenzopyrylium nucleus. These modifications include glycosylation of the 3-, 5-, 7-, or 3′-OH groups, followed by further chemical modifications including methylation, hydroxylation, and acylation.

The most common acyl modifications can be separated into two groups, addition of phenolic acids, including hydroxycinnamoyl derivatives, and addition of aliphatic acids. Regardless of the acyl group transferred, the enzymatic synthesis of acyl-modified anthocyanins appears to be carried out solely by members of the BAHD superfamily of plant acyltransferases (reviewed in [2]). The BAHD acyltransferase family comprises a large class of acyl-CoA utilizing enzymes involved in a diverse array of secondary metabolite modifications [3]. Large scale genomic sequencing projects have shown that the full complement of BAHD members can be quite large, even within a single plant species, consistent with the observation that plants contain numerous acyl-modified secondary metabolites [4].

In our goal to functionally characterize the remaining unidentified members of the *A. thaliana* BAHD (AtBAHD) family, we have combined reverse genetics with a biochemical genomics approach. As it was previously reported that this species synthesizes several acyl-modified anthocyanins [5], we investigated whether the enzyme(s) responsible for these modifications could be one of the yet uncharacterized members of the AtBAHD family. Here, we report the identification and characterization of *At3g29590* as a member of the AtBAHD family encoding the enzyme malonyl-CoA: anthocyanidin 5-*O*-glucoside-6″-*O*-malonyltransferase (At5MAT), responsible for synthesizing malonyl-modified anthocyanins in *A. thaliana*.

2. Materials and methods

2.1. Plant materials and treatments

Arabidopsis thaliana (L.) Heynh (ecotype Columbia) plants were grown on soil in a climate-controlled growth chamber [22 °C, 55% relative humidity, and 100 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR)] under either short (12 h light/12 h dark) or long (16 h light/8 h dark) days for up to 4–6 weeks. Selection and analysis of transgenic plants used in this study were performed as previously described [6]. All treatments were performed with 4–6-week-old rosette stage (non-bolting) plants of *A. thaliana* (ecotype Columbia). For the anthocyanin-inducing treatment, plants were switched from short to long day conditions.

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The T-DNA insertion line SAIL_1151_A03 (stock number N842367) was obtained from the European Arabidopsis Stock Center (NASC, Loughborough, United Kingdom) and analyzed for resistance to glufosinate-ammonium as described in [7]. In addition, a PCR-based assay was designed to detect the presence of the T-DNA insertion (see supplementary material for all primer sequences used in this study). For qualitative RT-PCR experiments, the gene *At1g49240* (*Actin8*) was used as a positive control. Plants homozygous for the T-DNA insert and segregating for glufosinate resistance were backcrossed for three generations to Col-0 plants. In every generation, 30 progeny were screened for presence of the T-DNA insert and a minimum of five positive plants based on the PCR assay were used as the pollen donors for backcrossing. Plants positive for a T-DNA insert after the third generation of backcrossing were allowed to self fertilize and their progeny were analyzed. Another mutant used in this study was *pap1-D* (NASC number N3884), the activation-tagged line for a MYB transcription factor resulting in constitutive anthocyanin accumulation [8].

2.2. Reagents

All solvents or reagents were molecular biology or reagent grade and were obtained from Fluka, Sigma-Aldrich, or Roth (Karlsruhe, Germany), unless otherwise noted.

The CoA thioesters cinnamoyl-CoA, caffeoyl-CoA and *p*-Coumaroyl-CoA were a gift from Dr. Silke Brand (MPI-ICE, Jena, Germany). Anthocyanin substrates used in the biochemical characterization of At5MAT were purchased from Extrasynthèse (Genay, France). These included pelargonin, malvin, kuromanin, ideain, and keracyanin. The *A. thaliana* native anthocyanins, cyanidin 3-*O*-[2″-*O*-(xylosyl)-6″-*O*-(*p*-*O*-(glucosyl)-*p*-coumaroyl) glucoside]5-*O*-glucoside (A6) and cyanidin 3-*O*-[2″-*O*-(2‴-*O*-(sinapoyl) xylosyl)6″-*O*-(*p*-*O*-(glucosyl)*p*-coumaroyl) glucoside]5-*O*-glucoside (A10) were purified from the SAIL_1151_A03 line (see below).

2.3. Enzyme assay and chemical analysis

The standard reaction mixture (final volume, 50 μL) consisted of 50 mM bis–Tris-propane, pH 7.1, 500 μM anthocyanin substrate, 500 μM malonyl-CoA, and the enzyme preparation. The reaction was started by the addition of the enzyme. After incubation at 25 °C for 1 h, the reaction was stopped by the addition of 50 μL of 0.5% (v/v) trifluoroacetic acid. For testing different CoA-esters, the anthocyanins A6 and malvin were used as co-substrates at a concentration of 500 μM each. Besides malonyl-CoA, the following CoA esters were also tested in the enzyme assay: caffeoyl-CoA, cinnamoyl-CoA, hexanoyl-CoA, butyryl-CoA, benzoyl-CoA, acetyl-CoA, and *p*-coumaryl-CoA.

Anthocyanin products were analyzed by reversed-phase HPLC using an Agilent 1100 system (Agilent Technologies): column, Supelcosil LC-18 (250 × 4.6 mm); injection volume, 50 μL; flow rate, 0.7 mL min^{-1}; solvent A, 0.5% (v/v) trifluoroacetic acid; solvent B, acetonitrile. The photodiode array detector was used in the range of 250–650 nm. Quantification of anthocyanins was achieved by detection at 520 nm using a calibration curve generated from authentic cyanin. Assay mixtures were analyzed with the following gradient: start 15% B, linear gradient from 15% B to 28% B in 13 min followed by a washing cycle.

Anthocyanins A6 and A10 were isolated and purified from leaves of *A. thaliana* mutant line SAIL_1151_A03 and were used as authentic samples after confirmation of their structures by mass spectrometry and NMR. Leaves (6 g) visibly accumulating anthocyanin pigments were homogenized with 10 mL of methanol:acetate:water (9:1:10). After centrifugation, the supernatant was concentrated in a rotary evaporator to 1.5 mL. The extract was separated by injection of 80 μL onto the HPLC: column, Hypersil 5ODS (250 × 10 mm); flow rate, 3.5 mL min^{-1}; solvent A, 0.5% (v/v) trifluoroacetic acid; solvent B, acetonitrile. The gradient was: start 10% B, linear gradient from 10% B to 30% B in 25 min, followed by a washing cycle. After individual anthocyanins were collected by use of a fraction collector, the fractions were evaporated to dryness and reconstituted in water (400 μL).

Chemical structures were determined by ESI-MS using a Hewlett-Packard (Avondale, PA, USA) HP 1100 HPLC coupled to a Micromass Quattro II (Waters, Micromass, Manchester, UK) tandem quadrupole mass spectrometer equipped with an electrospray (ESI) source [9]. Argon was used for collision-induced dissociations (CID) at 1.5×10^{-3} mbar and the collision energy was varied from 12 to 50 eV for fragmentation. Fixed product spectra (parent-ion scan) were recorded at higher cone voltage (typical setting 50–70 V) to increase the abundance of the observed parent ion (*m*/*z* 287).

2.4. Isolation and cloning of At3g29590 (At5MAT) cDNA and expression in E. coli

The open reading frame of *At3g29590* (accession NM_113880) was isolated from genomic DNA of Col-0 plants and was subcloned into the entry vector pDONR207 (Invitrogen, Karlsruhe, Germany) by introducing the required attB1 and attB2 recombination sites at the 5′ and 3′ ends in a two-step PCR process. Following a BP clonase© reaction and sequencing, the clone was used to generate the expression plasmid At3g29590-pH9GW via an LR clonase© reaction according to the manufacturer's protocols. The vector pH9GW is a derivative of the pET-T7 (28a) vector (Novagen, Madison, WI, USA) containing a 9× His-tag followed by a gateway attR cassette [10]. Expression and harvesting of the recombinant protein in BL21(DE3) *E. coli* cells was accomplished as previously described [11].

2.5. Purification of recombinant proteins

Isolation and purification of recombinant proteins were performed either on ice or in a refrigerated chamber operating at 8 °C. At5MAT protein containing a 9× His-tag was purified as described in [6]. All purification steps were analyzed by SDS–PAGE gel electrophoresis followed by Coomassie Brilliant Blue or silver staining of the gel. At5MAT protein concentration was determined as previously described [12] using bovine serum gamma globulin as the standard. Activity assays of the purified recombinant At5MAT enzyme were performed as described in [13] except that the activity of the enzyme was assayed with five different concentrations of each substrate at least six times in three replicates each (18 total), while keeping the second substrate at saturating conditions (defined as 10× the K_m). Activity was linear for the incubation time and protein concentration employed.

2.6. Construction of At3g29590 RNAi vector

In order to clone the At3g03480 RNAi construct, the GATEWAY© compatible binary vector pHellsgate8 for dsRNA production was obtained from CSIRO Plant Industry (Canberra, Australia) [14]. A 551 bp segment from the 3′ end of the *At5MAT* cDNA (position 776–1327) was amplified by PCR and the resulting fragment was cloned and placed into pDONR207, sequenced, and used for producing the destination vector pHellsgate-At3g29590 as described above for the expression construct. *Agrobacterium tumefaciens* strain GV3850 was used for the transformation of *A. thaliana* (ecotype Columbia) via floral vacuum infiltration [15]. Ninety independent T1 lines were chosen based on selection on kanamycin and presence of the transgene, and used in subsequent chemotyping analysis.

2.7. DNA and RNA analysis

Genomic DNA extraction, hybridization conditions for DNA gel blot analysis, RNA extraction, first strand cDNA synthesis, and primer validations for quantitative RT-PCR experiments were all performed as previously reported [6]. All primers were HPLC-purified and have efficiencies between 90% and 100%. In addition, the products of each primer combination were cloned and sequenced a minimum of three times to confirm the sequence of the amplicon.

Comparative quantification experiments included a minimum of three biological replicates with three technical replicates for each sample unless otherwise stated. The $\Delta\Delta C_t$ method of comparative quantification employing efficiency correction was used to judge the relative quantification of starting template for all genes of interest [16]. Data were not used if the non-template (H_2O) controls had an amplified product within 5 C_t values of the highest C_t for the true biological samples.

3. Results

3.1. Analysis of the AtBAHD family reveals a small clade of genes encoding putative anthocyanin acyltransferases

To identify potential anthocyanin acyltransferases in *A. thaliana*, we first performed a neighbor joining analysis of the