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Physiological and molecular analyses of chlorophyllase in sweet potatoes with different-colored leaves

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ABSTRACT

Chlorophyllase (Chlase) is a hydrophobic enzyme that catalyzes the hydrolysis of chlorophyll (Chl) to chlorophyllide (Chlide) and phytol. The objectives of this research were to study Chlase characteristics of sweet potatoes (*Ipomoea batatas*, Ib) with various leaf colors: yellow (CN1927), green (TN57), and purple (CYY8467). Large variations in Chl and anthocyanin (Ant) and carotenoid (Car) existed in all plants, and CYY8467 contained significantly higher Chl, Ant, and Car levels compared to TN57 and CN1927. Various plants exhibited different Chlase activities, and Chl *a* degradation by Chlase of CYY8467 was higher than those of TN57 and CN1927. Nevertheless, Chlase activity in CN1927 exhibited a higher substrate preference toward Chl *b* than Chl *a*. Expression of the Chlase gene was detected in all leaves; however, IbChlase mRNA was most strongly active and regulated in TN57. The open reading frame sequences of IbChlase in TN57, CN1927, and CYY8467 shared 95% identity, and an eight-nucleotide sequence of Chlase in CYY8467 was missing due to a frame-shift mutation of the Chlase gene leading to early termination of gene translation. A phylogenetic analysis of Chlase indicated that 30 plants diverged into three clades, and sequences from TN57 and CN1927 were clustered together in a clade of the Chlase2.

1. Introduction

Chlorophyll (Chl), anthocyanin (Ant), and carotenoid (Car) are the most important plant pigments. The amount of solar radiation that a leaf absorbs is determined mainly by Chl; thus, the foliar Chl content directly affects the photosynthetic potential and primary production (Filella et al., 1995). The Chl biosynthesis and/or degradation pathway influences Chl accumulation. More than one Chl degradation pathway has been elucidated in different physiological and developmental conditions, including chlorophyllase (chlorophyll chlorophyllidohydrolase, Chlase, EC 3.1.1.14), that degrades Chl to phytol, and chlorophyllide (Chlide), that leads to a rate-limiting step in the Chl-degradation pathway mediated by posttranslational regulation (Cheng et al., 2012). In addition, pheophytinase, which is responsible for converting Mg^{2+}-free Chl to pheophorbide, may lead to another Chl degradation pathway in senescing leaves (Schelbert et al., 2009). Both proposed pathways show that Chl is cleaved into several intermediate catabolites in chloroplasts. Previously, we found that leaves of sweet potato (Hsu et al., 2003) and *Machilus thunbergii* (Yang et al., 2003) use Chl → pheophytin → pheophorbide as the major route for Chl degradation, whereas leaves of banana might use Chl → Chlide → pheophorbide as the major route (Hsu et al., 2011). In addition, some biotic/abiotic factors also affect the degradation pathway (Hsu et al., 2003; Yang et al., 2003; Hsu et al., 2011; Huang et al., 2014).

Kariola et al. (2005) reported the biological functions of AtChlase1 in Chl degradation and cell damage. The Chl content is one of the determining factors of plant leaf color, which maintains a dynamic balance under continual biosynthesis and degradation in normal green plants, and the green-deficient or stay-green leaf color occurs as this balance is destroyed (Shi et al., 2009). We compared Chlase activities of seven selected evergreen plants and found that *Pachira macrocarpa* contained significantly higher Chlase activities than the others, and the activities were localized in the subcellular fraction of the chloroplast envelope (Chen et al., 2012). Accordingly, two full-length genes of Chlase isoforms were cloned from *P. macrocarpa*, which shared 84% identity throughout the entire amino acid sequences, and belonged to the same clade as *Arabidopsis* AtChlase1. Differential expression patterns of the two genes and protein levels of the Chlase isoforms from various tissues suggested that these two isoforms have different biological functions *in vivo* (Chen et al., 2014).

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Sweet potato is the world's fifth most important crop and is a major source of food and nutrition in developing countries (Food and Agriculture Organization, 2002). Various leaf colors of sweet potatoes grow under natural conditions, but the cause of its leaf color formation in terms of Chlase remains unclear, and research on characteristics of Chl metabolism in its leaves has not been reported. Chlase in the different leaves exhibits different abilities and specificities for Chl *a* and *b* degradation that play different roles in different parts of the leaf depending on the plant species. In view of this, contents of Chl and Ant, the contents of the major synthetic precursors in Chl biosynthesis, and the activities of Chlase that catalyze the biosynthesis and degradation of Chl in the leaves of sweet potatoes were determined in this study. We also cloned open reading frames (ORFs) of Chlase-complementary (c)DNA, studied their leaf expression patterns, and analyzed the evolution of Chlase genes among plant species. Our findings lay the foundation for researchers interested in physiological and molecular studies of Chlase, which is an essential enzyme involved in the Chl degradation pathway.

2. Materials and methods

2.1. Plant materials and culture practice

Three leafy vegetable sweet potato (*Ipomoea batatas* (L.) Lam) cultivars with green (TN57), yellow (CN1927), and purple leaves (CYY 8467) were gifts from Dr. Y.C. Lai, at the Department of Agronomy, Chiayi Agricultural Experiment Station, Chiayi, Taiwan. TN57 is the major sweet potato cultivar for food consumption in Taiwan. CN1927 was bred by World Vegetable Center in Shanhua, Tainan, Taiwan, and is suitable for vegetable-used purposes. CYY8467 was bred by Dr. Lai's group, and is used as a vegetable-consumption sweet potato. Cuttings about 40 cm in length were taken from sturdy vines, and cultivated in plastic boxes 60 cm long, 22 cm wide, and 15 cm deep, that contained a medium of sand, vermiculite, and loamy soil in a volume ratio of 2:1:1. Specimens were planted in November and December 2015 in a screen house at the Chinese Culture University, and evenly spaced at intervals of 50 cm to encourage similar growth rates and sizes. During the period of study, average day/night temperatures were 26/21 °C and average day length was 12 h. Plants were watered with half-strength Hoagland solution (Hoagland and Arnon, 1950) every other day to maintain optimal irrigation, and allowed to grow for 21 d before the following measurements were made.

2.2. Preparation of acetone powder and assay of Chlase activity

Leaves frozen in liquid nitrogen were ground up with a mortar and pestle and homogenized with pre-chilled acetone (−20 °C). After centrifuging the homogenate at 3000*g* and 4 °C for 5 min, the precipitate was collected. The acetone powder was dried with nitrogen and stored at −20 °C until use. To determine the activity of Chlase, an enzyme solution was prepared based on our previous study (Chen et al., 2014). Briefly, 100 mg of acetone powder was homogenized, and the supernatant, after centrifuging at 15,000*g* for 15 min, was used for the enzyme assay. The standard reaction mixture contained 0.1 ml of substrate (1 μmol/ml Chl *a* or *b*), 0.1 ml of the above supernatant, and 0.8 ml of reaction buffer containing 100 mM sodium phosphate (pH 7.0) and 0.24% Triton X-100. The mixture was incubated for 60 min at 30 °C. After reacting, 1 ml of the mixture was further mixed with 5 ml of a hexane/acetone (3:2, v/v) solvent to eliminate the interference of Chl. The product, Chlide a or b, in the acetone phase was determined with a Hitachi U-2000 spectrophotometer (Tokyo, Japan) using an extinction coefficient of 74.9 mM^{-1} cm^{-1} at 667 nm or 47.2 mM^{-1} cm^{-1} at 650 nm for Chlide a or b, respectively. One unit of Chlase a or b degradation activity was defined as the amount of enzyme needed to catalyze the production of 1 μmol of Chlide a or b per minute.

2.3. Measurements of Chl, Ant, and Car contents

Determination of the Chl content was based on a SPAD (Soil Plant Analysis Development) analyzer (SPAD-502 Chlorophyll Meter, Konica Minolta, Tokyo, Japan). The relative amount of Chl in a leaf was measured by the optical density differences between two wavelengths of 650 and 940 nm. The Ant content was measured according to the protocol of Mancinelli et al. (1975). A mixture of 80% methanol containing 1% HCl as a solvent was used to extract the powder samples. The mixture was then centrifuged at 3000 rpm and 4 °C for 5 min, and the supernatant was used to measure the absorbance at 536 and 650 nm. The Ant content was calculated as (A535 − A650) / fresh mass (FM, g). The Car concentration was determined according to the protocol as described by Sumanta et al. (2014). Fresh powder sample was extracted with methanol followed by centrifuging at 10,000 rpm at 4 °C for 15 min. The absorbance of supernatant was detected at 470, 652, and 665 nm. The concentration of Car was calculated as $(1000A_{470} - 1.63\ (16.72A_{665} - 9.16A_{652}) - 104.96\ (34.09A_{652} - 15.28A_{665}))$ / 221 (μg/ml).

2.4. RNA extraction, complementary (c)DNA synthesis, and real-time qualitative polymerase chain reaction (qPCR)

Total RNA was isolated from 0.1 g of young leaves of sweet potato with a Qiagen RNeasy Plant Mini Kit (Valencia, CA, USA), and then poly (A)+ messenger (m)RNA was extracted from total RNA with a Qiagen Oligotex Mini Kit according to the vendor's instructions. Concentrations of total RNA and mRNA were determined with a NanoDrop ND-1000 spectrophotometer (Paris, France) at 260 nm. A real-time qPCR analysis was employed to quantify and validate relative changes in expressions of Chlase genes. First-strand cDNA synthesis was performed with an Oligo (dT) primer using RETROscript Reverse Transcription for the real-time PCR Kit (Ambion, Austin, TX, USA). cDNA was diluted 1:5 for the real-time PCRs which were carried out in 384-well plates in a Light Cycler 480 (Roche, Basel, Switzerland). Gene-specific primers designed from a partial sequence of Chlase from a transcriptome dataset of sweet potato (Chen et al., 2016a) for IbChlase (F: 5′-ATGCACAGCCCAAATCACAAACT-3′ and R: 5′-TTCCTCAGTCCTTTGA TCTTGGC-3′) were used in the real-time qPCR. The correct size of the amplified region for each primer was checked by agarose gel electrophoresis. Each real-time qPCR (20 μl) contained 1 μl of diluted cDNA, 9 μl of water, 2 μl of primer mix, 4 μl of SYBR green I, 1.6 μl MgCl2, 0.4 μl of the enzyme mix, and 2 μl of resolution solution (Roche RNA Amplification Kit). The amplification program consisted of one cycle at 95 °C for 5 min for pre-incubation, followed by 40 cycles at 95 °C for 10 s, 60 °C for 10 s, and 72 °C for 20 s. After amplification, a melting-curve analysis was run using the program of one cycle at 95 °C for 5 s, 65 °C for 10 s, and 95 °C with 0 s held in the step-acquisition mode, followed by cooling to 40 °C for 30 s. Three replicates were performed for each cDNA sample, and template-free and negative controls were used. To normalize the total amount of cDNA in each reaction, the actin7 gene of *I. batatas* (Chen et al., 2016b) was co-amplified as the internal control using primers of actin7F (5′-TTTGCTGGTGATGATGC-3′) and actin7R (5′-GCAACATACATGGCAGG-3′). Data were analyzed with the PCR efficiency correction using Light Cycler 480 Relative Quantification software vers. 1.01 (Roche) based on relative standard curves describing the PCR efficiencies of the Chlase and internal reference genes. These relative RNA quantities of RNA samples are presented as “expression” values in Fig. 3, and these values allowed comparisons of relative RNA amounts among tissues.

2.5. Molecular cloning of cDNA of IbChlases

To clone Chlase genes from sweet potato, a 5′ and 3′ RLM-RACE (rapid amplification of cDNA ends) was conducted according to the manufacturer's instructions (GeneRacer™ Kit, Invitrogen, CA, USA). Primers used in the 5′RACE (GSOR: 5′-AGAGCTAGCCCGAATGCTACTTT