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Isolation and characterization of the betalain biosynthesis gene involved in hypocotyl pigmentation of the allotetraploid *Chenopodium quinoa*

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In quinoa seedlings, the pigment betalain accumulates in the hypocotyl. To isolate the genes involved in betalain biosynthesis in the hypocotyl, we performed ethyl methanesulfonate (EMS) mutagenesis on the CQ127 variety of quinoa seedlings. While putative amaranthin and celosianin II primarily accumulate in the hypocotyls, this process produced a green hypocotyl mutant (*ghy*). This MutMap+ method using the quinoa draft genome revealed that the causative gene of the mutant is *CqCYP76AD1-1*. Our results indicated that the expression of *CqCYP76AD1-1* was light-dependent. In addition, the transient expression of *CqCYP76AD1-1* in *Nicotiana benthamiana* leaves resulted in the accumulation of betanin but not isobetanin, and the presence of a polymorphism in *CqCYP76A1-2* in the CQ127 variety was shown to have resulted in its loss of function. These findings suggested that *CqCYP76AD1-1* is involved in betalain biosynthesis during the hypocotyl pigmentation process in quinoa. To our knowledge, *CqCYP76AD1-1* is the first quinoa gene identified by EMS mutagenesis using a draft gene sequence.

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1. Introduction

Quinoa (*Chenopodium quinoa* Willd) is an allotetraploid species ($2n = 4x = 36$ with a genome size of 1448 Mbp) comprised of two distinct genomes, A and B [1]. It is a native grain to the Andean highlands in South America and has become an attractive pseudocereal in recent years. Quinoa seeds are a source of diverse nutrition, including vitamins, natural antioxidants, and high levels of essential amino acids [2]. Quinoa also exhibits a strong tolerance to environmental stresses [3]. In 2013, the Food and Agriculture Organization of the United Nations stated that quinoa is a cereal that could potentially mitigate a global food crisis [4].

Betalains are tyrosine-derived red-violet and yellow pigments found exclusively in Caryophyllales plants, including red beet and quinoa. Betalain has high antioxidant activity [5] and has been shown to be involved in environmental and biotic stress in plants [6,7]. Betalain biosynthesis begins with the hydroxylation of L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA) by both *CYP76AD1* and *CYP76AD6*, which are redundant enzymes in the process [8]. L-DOPA is then converted to betalamic acid by DOPA 4,5-dioxygenase (DODA) [9], or to cyclo-DOPA by *CYP76AD1* [10]. Betalamic acid can further spontaneously condense with amino acids or other amine groups to form yellow fluorescent betaxanthin pigments [11], or spontaneously condense with cyclo-DOPA to form red betacyanin pigments [12]. Betalain-related glucosyltransferases catalyze the 5-O glucosylation of cyclo-DOPA [13] or, alternatively, the 5-O or 6-O glucosylation of betanidin [14]. However, the genes involved in quinoa betalain biosynthesis have yet to be identified.

The establishment of a gene analysis method using next generation sequencing (NGS), such as MutMap [15], has facilitated the identification of numerous single-nucleotide polymorphisms

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(SNPs). In 2016, the draft genome sequence of quinoa was determined [16]. Building on this earlier work, the precise genome of quinoa was also recently revealed [17,18]. These genomic data enable for the identification of mutations, such as those in EMS mutants. Thus, genetic identification using NGS may be a useful tool for quinoa that cannot be transformed.

In this study, we focused on pigmentation in the quinoa hypocotyl. Specifically, in order to isolate a betalain biosynthetic gene, we obtained two lines of green hypocotyl mutants (*ghy*) by EMS mutagenesis. Using the MutMap+ method [19], we then revealed that the causative gene of the *ghy* mutant is *CqCYP76AD1-1*. In this report, we also analyze the functions of this gene in quinoa hypocotyl.

2. Materials and methods

2.1. Quinoa plant materials, growth conditions, and mutagenesis

Seeds of the Kd [16] and CQ127 varieties of *Chenopodium quinoa* Willd (quinoa) were obtained from Kyoto University and the USDA, respectively. Quinoa seeds were sown in a cell tray and grown at 23 °C with a neutral photoperiod (12 h light/12 h dark) in a phytotron. After three weeks, the seedlings were transferred in 5 L plant pots and grown in a greenhouse.

We entrusted the EMS mutagenesis to Inplanta Innovations (Yokohama, Japan) and obtained approximately 2000 mutagenized seeds (M1 seeds) of the CQ127 variety. The mutagen-administered seeds were sown to raise M1 progeny and were grown until the M3 generation was produced. The M3 progeny were then screened for hypocotyl mutations.

2.2. Identification of candidate mutations by MutMap analysis

The libraries for NGS were constructed with the TruSeq library preparation kit (Illumina, San Diego, CA), and paired-end sequencing was performed for 150 cycles on an Illumina Hiseq X platform. To filter out low-quality short reads, short reads where >10% of sequenced nucleotides exhibited a phred quality score of <30 were excluded from the analysis.

The candidate SNPs were then analyzed by the MutMap pipeline ver. 1.4.4 (http://genome-e.ibrc.or.jp/home/bioinformatics-team/mutmap). In the MutMap pipeline, alignment was performed by BWA [20], and alignment files were converted to SAM/BAM files using SAMtools [21]. In this pipeline, the short reads obtained from red bulk (wild-type bulk) were aligned to the "green bulk reference sequence," which was developed by replacing nucleotides of the public quinoa reference genome (Cqu_r1.0 http://quinoa.kazusa.or.jp/) with the SNPs (showing a SNP index > 0.9) detected by aligning the short reads obtained from the green bulk (mutant-type bulk). The SNP indexes for the red bulk were then calculated at all SNP positions.

2.3. Molecular cloning

Total RNA was extracted with the RNeasy Plant Mini kit (Qiagen, Valencia, CA) and treated with RNase-free DNase I (Qiagen) to eliminate genomic DNA. First-strand cDNA was synthesized from 500 ng of total RNA using the TaKaRa RNA PCR kit (AMV) Ver. 3.0 (TaKaRa, Kusatsu, Japan) with oligo(dT) primers. Genomic DNA was extracted with the DNeasy Plant Mini kit (Qiagen). We obtained the full-length ORF and genome sequence of *CqCYP76AD1-1* (XM_021913610), *CqCYP76AD1-2* (XM_021876908), *CqDODA1* (XM_021913611), and *CqCDOPA5GT* (XM_021892614) from the NCBI gene database. *AcGFP1* was used as a vector control (TaKaRa).

2.4. Quantitative real-time PCR analysis

First-strand cDNA was synthesized from 500 ng of total RNA using a High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) with random primers. Quantitative real-time PCR was carried out using ABI7300 (Applied Biosystems) with the KAPA SYBR FAST ABI Prism qPCR Kit (Kapa Biosystems, Woburn, MA). The amplification conditions were 95 °C for 5 min, followed by 40 cycles of 5 s at 95 °C and 30 s at 63 °C, with plate reading after each cycle. The primers specifically amplified the corresponding *CYP76AD1-1* gene. *CqMON1* was used as an internal control in each experiment [22]. Primer pairs are listed in Supplementary Table 1.

2.5. Reverse transcriptional PCR (RT-PCR) analysis

Synthesis of first-strand cDNA was as described above. RT-PCR was performed using a GeneAtlas 322 (Astec, Fukuoka, Japan). The procedure for amplification of the *CqCYP76AD1-2* transcript comprised an initial denaturation at 94 °C for 2 min, followed by 35 cycles of 94 °C for 15 s, 55 °C for 30 s, and 68 °C for 2 min. Primer pairs are listed in Supplementary Table 1.

2.6. Transient expression in Nicotiana benthamiana

Expression constructs were transformed into *Agrobacterium tumefaciens* GV3101 by the triparental mating method [23]. Transformed *Agrobacterium* suspensions were infiltrated into the leaves of 5- to 6-week-old *N. benthamiana* plants, as described previously [24]. Infiltrated plants were cultivated in a growth chamber at 23 °C with 60% humidity under long day (LD) conditions (16 h light/8 h dark).

2.7. Overexpression in quinoa hairy root

Quinoa seeds were germinated on MS plates containing 4.3 g/L MS (Wako, Tokyo, Japan), 1.5% sucrose, and 2.5 g/L Gelrite (Wako), and kept at 23 °C under LD conditions for 10 days until the cotyledons were fully expanded. *R. rhizogenes* (ATCC15834) were transformed by the freeze and thaw method [23] and were cultured in 3 mL LB liquid medium, with 50 mg/L kanamycin for one day at 26 °C. The hairy root induction and selection were performed in accordance with Ron et al. [25].

2.8. Plant pigment chemical analysis

Pigments were extracted from quinoa hypocotyl, quinoa hairy roots, and *N. benthamiana* leaves. Pigments were analyzed as described previously [10].

A Shimadzu LC-20AD apparatus (Shimadzu, Kyoto, Japan) was used for analytical HPLC separations. Samples were separated on a 5C18-AR-300 column (250 × 100 mm; Nacalai Tesque, Kyoto, Japan), and linear gradients were established from 0% B to 50% B in 60 min using H_2O with 0.05% trifluoroacetic acid (TFA) (solvent A) and acetonitrile with 0.05% TFA (solvent B). The flow rate was 1 mL min^{-1}, operated at 25 °C and the eluate was monitored in terms of absorbance at 536 nm for betacyanins.

3. Results

3.1. Pigment analysis of quinoa hypocotyl

We analyzed the pigmentation of the Kd and CQ127 varieties of quinoa. The hypocotyl of both varieties showed a red pigmentation (Fig. 1A). The leaves of three-month-old Kd and CQ127 varieties turned from green to red and yellow, respectively (Supplementary

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