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Effects of cold stress on transcripts and metabolites in tartary buckwheat (*Fagopyrum tataricum*)

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ABSTRACT

Plants recognize multiple environmental signals that lead to substantial changes in the regulation of primary and secondary metabolism in order to adapt to environmental stresses. In this study, we investigate the effects of cold on the metabolome and transcriptome of tartary buckwheat, focusing on the phenylpropanoid biosynthetic pathway. Using RNA-sequencing analysis of the cold-regulated transcriptome of buckwheat, we identified several phenylpropanoid biosynthetic transcripts that accumulated in response to cold. To confirm the transcriptome data, we analyzed the expression of the phenylpropanoid biosynthetic transcripts in cold-treated buckwheat and showed that most of the phenylpropanoid biosynthetic transcripts were upregulated in cold-treated buckwheat seedlings with the exception of *FtDFR*. From the metabolic profiling based on the GC-TOFMS analysis, we show that most of the sugars and their derivatives significantly increase in response to cold, while some of amino acids and their derivatives decrease in cold-treated plants. Some organic acids derived from the tricarboxylic acid (TCA) cycle increased in the cold-treated plants compared with in the plants grown at 25 °C. In particular, the contents of anthocyanins and proanthocyanidins were significantly increased by cold treatment. In summary, these results indicate that the metabolome and transcriptome of tartary buckwheat are extensively affected by cold stresses.

1. Introduction

Plants live in restricted spaces that are constantly exposed to various environmental stresses such as cold, drought, high salinity, high light/UV and heavy metals. During these stressful conditions, plants biosynthesize specialized metabolites to adapt to these environmental stresses. Many phenylpropanoid compounds are induced in plants by abiotic stresses (Dixon and Paiva, 1995). Wounding stimulates the accumulation of coumestrol, coumarin, chlorogenic acid and polyphenolic barriers such as lignin (Hahlbrock and Scheel, 1989; Bernards and Lewis, 1992). Wound-induced polyphenolic barriers may act directly as defense compounds. UV induces specific flavonol glycosides and sinapate esters in *Arabidopsis* and rutin and quercetin in buckwheat (Hectors et al., 2014; Huang et al., 2016). Anthocyanins and some flavonols accumulated in response to high light, cold and drought (Li et al., 2013, 2015; Nakabayashi et al., 2014).

Cold has a significant role in the regulation of primary and secondary metabolism in plants (Guy et al., 2008; Cook et al., 2004). Cook et al. (2004) used a GC-time of-flight MS metabolic profiling approach to identify which metabolome was altered in response to cold (Cook et al., 2004). Of the 434 metabolites monitored by this technique, 325 (73%) were found to increase in *Arabidopsis* Wassilewskija-2 (Ws-2) during cold conditions. The 114 metabolites (35%) including the amino acid proline and the sugars glucose, fructose, galactinol, raffinose and sucrose increased at least 5-fold in cold-treated plants. Cold affects the general phenylpropanoid and anthocyanin synthesis in apple skin, blood orange fruit, purple kale, buckwheat and maize seedlings by inducing the expression of flavonoid biosynthetic genes (Ubi et al., 2006; Crifo et al., 2012; Zhang et al., 2012; Li et al., 2015; Christie et al., 1994). The expression of five anthocyanin biosynthetic genes including

Abbreviations: ANR, anthocyanidin reductase; ANS, anthocyanin synthase; C4H, cinnamate 4-hydroxylase; CHI, chalcone isomerase; CHS, chalcone synthase; 4CL, 4-coumarate:CoA ligase; DFR, dihydroflavonol reductase; DPPH, 2,2-diphenyl-1-picrylhydrazyl; F3H, flavanone 3-hydroxylase; F3'H, flavonoid 3'-hydroxylase; FLS, flavonol synthase; GC-TOFMS, gas chromatography-time of flight mass spectrometry; 3GT, flavonoid 3-O-glucosyltransferase; HPLC, high-performance liquid chromatography; LAR, leucoanthocyanidin; PA, proanthocyanidin; PAL, phenylalanine ammonia-lyase; RT, 3-O-rhamnosyltransferase; TCA, tricarboxylic acid

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MdCHS, *MdF3H*, *pDFR*, *MdANS* and *pUFGluT* was enhanced by cold treatment with the accumulation of anthocyanins in apple skin during ripening (Ubi et al., 2006). The anthocyanin levels in cold treated purple kale were approximately 50-fold higher than those in plants grown in a greenhouse (Zhang et al., 2012). The expression of the anthocyanin biosynthetic genes *BoC4H*, *BoF3H*, *BoDFR*, *BoANS* and *BoUFGT* and the transcription factor *BoPAP1* were enhanced by cold in purple kale. The anthocyanin content of blood orange fruit exposed to cold sharply increases more than 8-fold compared to that observed in the untreated samples (Crifo et al., 2012). The expression of anthocyanin biosynthetic genes (*CM1*, *PAL*, *CHS*, *DFR*, *ANS* and *UFGT*) increased after 3–6 days of cold storage, reconfirming previous data showing that the anthocyanin biosynthesis is regulated by the cold signaling pathway.

Some phenylpropanoids and anthocyanins are the major components of secondary metabolites that are affected by cold (Olsen et al., 2009; Li et al., 2015). Phenylpropanoids are diverse group of compounds derived from the carbon skeleton of the amino acid phenylalanine that is an end product of the shikimate pathway (Herrmann and Weaver, 1999; Vogt, 2010; Fraser and Chapple, 2011). Phenylalanine is converted into 4-coumaroyl-CoA by three enzymes, i.e., phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H) and 4-coumarate:CoA ligase (4CL), in the general phenylpropanoid pathway (Supplementary Fig. S1). Chalcone synthase (CHS) catalyzes 4-coumaroyl-CoA to produce naringenin chalcone that is catalyzed into naringenin through a stereo specific isomerization reaction by chalcone isomerase (CHI). In next step, flavanone 3-hydroxylase (F3H) converts naringenin to dihydrokaempferol that can be catalyzed into dihydroquercetin by flavonoid 3′-hydroxylase (F3′H). Dihydroquercetin serves as a common precursor that can be catalyzed by either flavonol synthase (FLS) to form several flavonols or dihydroflavonol reductase (DFR) in the first step for anthocyanin biosynthesis (Winkel-Shirley, 2002). Anthocyanin synthase (ANS) then catalyzes the common step in the anthocyanin and proanthocyanidin (PA) biosynthetic pathway. Cyanidin is converted into cyanidin-O-glucoside and cyanidin-O-rutinoside by flavonoid 3-O-glucosyltransferase (3GT) and 3-O-rhamnosyltransferase (RT), respectively. Leucoanthocyanidin reductase (LAR) and anthocyanidin reductase (ANR) can produce proanthocyanidins (PAs) such as catechin and epicatechin (Abrahams et al., 2003; Xie et al., 2003; Matsui et al., 2016).

Tartary buckwheat (*Fagopyrum tataricum*) is an annual plant in the family Polygonaceae that has been considered to be an alternative crop or minor cereal in Asia, Europe, North America and South Africa. Recently, tartary buckwheat has become known as a rich source of numerous health-benefiting compounds such as vitamins B1, B2 and B6, rutin, quercetin, chlorogenic acid, anthocyanins and proanthocyanidins (Bonafaccia et al., 2003; Suzuki et al., 2005; Kim et al., 2007). In particular, rutin is the predominant flavonoid in tartary buckwheat that reaches 50–60 mg g^{-1} dry weight (Kim et al., 2006). Despite the nutritional value of tartary buckwheat, the transcriptome and metabolome responses to cold stress in this plant are still poorly understood. Buckwheat is grown under almost limited environmental conditions during growth and developmental stages, especially at the early stage of development and flowering. The buckwheat is highly sensitive to frost at the early developmental stage. The seeds exposure to cold at 2 °C for 3, 5, and 7 h exhibited decreased germination rates from 70% to 90%. The value of average plant height was decreased from 19% to 30% in cold-treated buckwheat compared with control buckwheat (Kumar and Srivastava, 2015). To ensure their survival, it is important to understand that how plant control the balance between tolerance and growth during stress exposure. In this study, we identified the changes that occur in the metabolome and transcriptome of tartary buckwheat in response to cold, focusing on the flavonoid biosynthetic pathway. We studied the dynamic transcriptional levels of the phenylpropanoid biosynthetic genes to cold. By analyzing both the primary and secondary metabolites of tartary buckwheat with or without cold, we showed metabolic responses to environmental stresses.

2. Materials and methods

2.1. Plant material and cold treatment

Tartary buckwheat cultivar “Hokkai T8″ was provided by the Hokkaido Agricultural Research Center (Hokkaido, Japan). Dehulled tartary buckwheat seeds were sterilized with 70% ethanol for 30 s and 4% (v/v) bleach solution for 15 min, and then rinsed several times in sterile water. These seeds were grown on agar plates containing sucrose-free half Murashige and Skoog (1/2 MS) medium with 2.5 mM MES, pH 5.7, and 0.8% agar at 25 °C with 16 h photoperiod. The light intensity was ~150 μmol m^{-2} s^{-1}. Six-day-old light-grown plants were incubated at 4 °C for varying periods with white fluorescent light for cold treatments. The samples were immediately frozen in liquid nitrogen and then stored at -80 °C for RNA isolation or freeze-dried for high-performance liquid chromatography (HPLC) analysis.

2.2. Illumina sequencing of the transcriptome

Total RNA was isolated from the frozen seedlings of tartary buckwheat using an RNeasy Mini Kit (Qiagen, Valencia, USA) and cleaned by ethanol precipitation. We removed the rRNAs in the total RNA using a ribo-zero rRNA removal kit (Epicentre, RZPL11016) and constructed a cDNA library for RNA sequencing using a TruSeq stranded total RNA sample prep kit-LT set A and B (Illumina, RS-122-2301 and 2302) according to the manufacturer’s instructions (Illumina, San Diego, CA, USA). The cDNA library was sequenced in 76 bp length paired-end (PE) reads in an Illumina NextSeq500 sequencer (Illumina Inc., San Diego, CA, USA) to produce 69,570,892 raw sequencing reads.

2.3. De novo assembly and annotation of the tartary buckwheat transcriptome

The quality-trimmed reads of tartary buckwheat RNAs were assembled as contigs of the tartary buckwheat transcriptome using the Trinity software package (http://trinityrnaseq.github.io) (Haas et al., 2013). The characteristic properties including the N50, average, maximum, and minimum lengths of the assembled contigs were calculated using Transrate software (http://hibberdlab.com/transrate) (Smith-Unna et al., 2016). We clustered the tartary buckwheat transcriptome contigs based on sequence similarity using CD-HIT-EST software (http://weizhongli-lab.org/cd-hit) (Fu et al., 2012). To infer the biological functions of tartary buckwheat transcripts, we performed a homology search of the transcripts in a number of public protein and nucleotide databases. The functional category distributions of tartary buckwheat transcripts in terms of Gene Ontology (GO) and COG were evaluated using the results of the homology search. COG functional category information attached to the COG proteins that fit the search parameters was used to determine the for determining COG functional category distribution, and GO information attached to the hit UniProt proteins was collected and re-analyzed using the WEGO tool (Ye et al., 2006) in terms of the level for the three GO categories.

2.4. Differentially expressed gene analysis

To quantify tartary buckwheat transcript expressions, we aligned preprocessed quality-trimmed reads on the tartary buckwheat transcript sequences and calculated the expression values with the aligned read counts for each transcript. Bowtie2 software (Langmead and Salzberg, 2012) was used to align the quality-trimmed reads on the transcript sequences, and eXpress software (Roberts and Pachter, 2013) was used to evaluate gene expression, in terms of fragments per kilobase of exon per million mapped fragments (FPKM) from the aligned results. The FPKM method provides a comparison between genes within