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Acid α-galactosidase is involved in D-chiro-inositol accumulation during tartary buckwheat germination

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Abstract

Tartary buckwheat seed and especially its sprouts are rich in D-chiro-inositol (DCI). The research was to evaluate when DCI was most accumulated in tartary buckwheat sprouts. In addition, we explored the activity and expression pattern of α-galactosidase during tartary buckwheat seed germination. The results showed that DCI contents steadily increased at early stage of germination and reached the highest level of 33.42 μg/seed at 24 h during the 72 h trail. However, the total fagopyritol contents sharply decreased from 214.6 μg/seed to 46 μg/seed at the end of the germination. The activity of acid α-galactosidase increased gradually to the peak of 0.36 nkat/seed at 24 h after the primed seed imbibition. We cloned the gene fragment of α-galactosidase in tartary buckwheat for the first time. The deduced amino acid sequence is 93% identical to that of *P. vulgaris*. The quantitative PCR result of gene expression pattern was consistent with its enzyme activity during seed germination.

Keywords: tartary buckwheat; D-chiro-inositol; germination; α-galactosidase; gene expression

Introduction

Tartary buckwheat (*Fagopyrum tataricum*) is a functional food, widely cultivated all over the world including Asia and southwest of China. As a functional food *F. tataricum* has been receiving much attention for its healing effects over chronic diseases for the long time [1]. It has been illustrated that intragastric administration of buckwheat concentrate effectively lowered serum glucose concentrations in streptozotocin-diabetic rat [2]. In humans, buckwheat has a therapeutic potential against hyperglycemia and diabetes mellitus [3].

D-chiro-inositol (DCI), a naturally occurring isomer of myo-inositol, is the main active nutritional ingredient in buckwheat. It acts as a component of a putative insulin mediator, a galactosamine D-chiro-inositol with an insulin like bioactivity [4]. DCI increase insulin sensitivity and decrease plasma glucose in obese rhesus monkeys with spontaneous insulin resistance [5]. In humans, non-insulin-dependent diabetes mellitus (NIDDM) has also been associated with decreased urinary DCI excretion [6]. Therefore, DCI has great potential to work as an adjunctive drug in the treatment of insulin resistance ailments such as type 2 diabetes and polycystic ovary syndrome [7].

Buckwheat is an excellent dietary source of DCI in the form of its a-galactosides, fagopyritols, that accumulate in embryo tissues of seeds [8,9]. There are several form of fagopyritols accumulated in buckwheat seeds [10]. However, DCI exists as its galactosyl derivatives limits the nutritional value of buckwheat seed [8]. Previous research has demonstrated that germination may have the potential to improve the nutritional value of the grain and can effectively reduce antinutrients in cereals and legumes [11].

Seeds have a high demand for energy during early germination. Raffinose family oligosaccharides (RFOs), which are ubiquitous in plant seed and are rapidly mobilized by α-galactosidase during seed germination to provide energy [12]. RFOs are important for early germination of plant. The inhibition of raffinose oligosaccharide breakdown delayed pea seeds germination indicating that galactose is an important component during germination [13]. Uniquely, buckwheat seeds accumulate small amounts of RFOs but large amounts of fagopyritols, more than 40% percent of total soluble carbohydrates, which can be hydrolysed by the α-galactosidase in vivo , releasing galactose and the free DCI [14]. Seed priming is the technique which is commonly used to improve germination behavior and seedling emergence [15]. The application of seed priming will induce the synchronization of physiological and biochemical changes during seed germination [16].

Galactosidase can be divided into two types depending on their optimal pH for activity. The acidic α-galactosidases are most likely active in the acidic environment of vacuoles

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while the alkaline forms probably catalyze galactose release in the more neutral or alkaline cytoplasm [17]. The acidic α-galactosidases prefer raffinose as the substrate in comparison with the alkaline form which shows a higher affinity for stachyose [18,19]. In germinating legume seeds, α-galactosidase plays a role in the mobilization of RFOs [20]. The characterization and cloning of α-galactosidase during germination have been studied in other plant [21–23]. The activity of α-galactosidase and the expression pattern of the gene especially during tartary buckwheat germination remain unknown.

Although some studies have been carried out to improve DCI contents in buckwheat sprouts, the effects of seed priming on DCI accumulation in tartary buckwheat sprouts have never been reported. Additionly, we cloned the gene fragment of α-galactosidase of tartary buckwheat and further tested the gene expression during seed germination by the qPCR method.

Material and methods

Tartary buckwheat seed germination

Tartary buckwheat seeds were purchased from Sichuan province of Southwest China and stored at −20°C. The following germination method was adopted as we reported before [24]. The seeds were surface sterilized with 10% (v/v) of sodium hypochlorite for 3 h and then washed. Then the seeds were mixed with sand containing 4% (v/w) water, sealed in plastic box and primed in darkness at 15°C for 48 h. After treatment, seeds were washed under tap water and dried to the original moisture content determined by weighing with forced air under shade at 27 ±3°C for 2 days. The primed seeds were spread thinly on petri dishes containing layers of wet filter paper and initiated to germinate in the dark (25°C for 72 hours). Seed samples were collected at 12-hour intervals from 0 to 72 hours after imbibition, immediately frozen in liquid nitrogen and the samples were stored at −80°C for further use.

Analysis of DCI and total fagopyritol content

Phenyl R-D-glucoside, trimethylsilylimidazole (TMSI) and pyridine were purchased from Sigma-Aldrich (Shanghai, China), DCI standards were purchased from Wako Pure Chemicals Industries, Ltd. (Osaka, Japan). Fagopyritol standards were extracted from seed buckwheat (*Fagopyrum esculentum* L.). The DCI and total fagopyritol content were performed according to the procedure of Yang and Ren [25] with slight modification. Three replications of 10 seeds each were blended for 5 min using homogenizer with 20 ml of ethanol/water (1:1, v/v) containing 10 mg of phenyl R-D-glucoside as internal standard. The homogenate was centrifuged at 12 000 *g* for 10 min at room temperature, supernatant was removed, and the residue was re-extracted two times with 10 ml of ethanol/water (1:1, v/v) for 5 min and recentrifuged. An aliquot of the combined extracts was filtered through 0.22 μm film, transferred to silylation vials, and evaporated to dryness in a stream of nitrogen gas at 70°C water bath.

Extract residues were kept overnight in a desiccator over phosphorus pentaoxide to remove traces of water. Dry residues were derivatized with a silylation mixture (TMSI/pyridine, 1:1, v/v) in silylation vials at 70°C for 30 min, cooled, and analyzed by gas chromatograph system (GC-7900, Shanghai, China) equipped with a FID detector and a HP-5 capillary quartz column (50 m × 0.25 mm, 0.25 μm film thickness). The initial column temperature was 150°C, which increased gradually up to 200°C by the velocity of 3°C/min. Subsequently, it was gradually increased to 325°C by the velocity of 7°C/min, and then maintained for 20 min. The inlet temperature was 335°C and detector temperature was 350°C. The carrier gas nitrogen was at 1.0 ml/min (measured at 30°C). The injection volume was 1 μl. DCI and the fagopyritols were identified by GC retention times identical to the standard.

DCI and total fagopyritols contents were quantified based on standard curves: the ratios of the area of signals for each known compound to the area of the signal for phenyl R-D-glucoside, the internal standard, were plotted against known amounts of each compound [20]. Amounts below the level of detection are presented as zero.

Assay on α-galactosidase activity

Three replications of 10 seeds each were ground in a glass homogenizer in extraction buffer (50 mM HEPES-NaOH, pH 7.4). Homogenates were centrifuged at 10 000 *g* for 20 min at 4°C, the supernatants were stored at 4°C prior to assay of α-galactosidase activity. The enzyme activity of α-galactosidase was determined as previously described [26]. The quantitative analysis of its activity was measured by detection the p-nitrophenol released from p-nitrophenyl-α-D-galactopyranoside (pNPGal). The reaction mixtures consisted of 0.9 ml substrate (3 mM pNPGal in 100 mM NaAc buffer, pH 5.0) and 0.1 ml of suitably diluted enzyme preparation. The reaction was terminated by the addition of 3 ml of 3% Na_2CO_3 after incubation for 15 min at 37°C and the quantity of p-nitrophenol released was measured at 410 nm. Blanks were prepared by adding the enzyme extract after Na_2CO_3. One unit of enzymatic activity, nkatal (nkat) was defined as the amount of activity that released one nmole of p-nitrophenol per second at pH 5.0 and 37°C. The enzyme activity was expressed in units per seed (nkat/seed). The data presented for all α-galactosidase activity determinations are mean values of triplicate assays in which the standard deviations were always smaller than 10%.

Cloning and sequence analysis of FtaGAL in buckwheat

As the buckwheat belongs to dicotyledon , the alignment of the amino acid sequence of α-galactosidase among various dicotyledonous plant species was done with Clustal X software [27]. The conservative sequences were used for designing two degenerate PCR primers, GALF: 5′-TGGG(G/A)(A/G)T (G/A)GA(C/T)TA (C/T) T (G/A)AA(A/G)TATG-3′ and GALR: 5′-TC(G/A)A(A/G)C AT (A/G) TC(G/ A) GG(A/G) TC (A/G) TTCC-3′.

Seed samples were powdered in liquid nitrogen with mortar and pestle and the total RNA was extracted using Trizol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Less than 500 ng RNA

was used for the RT-PCR as specified in the RNA PCR Kit (AMV) Ver.3.0 (TaKaRa, Japan). The oligo dT-adaptor primer was used for cDNA synthesis at 42°C, 60 min. The PCR amplification was carried out with above-described degenerate primers. The amplification profile was one cycle at 93°C for 3 min, followed by 35 cycles at 93°C for 15 s, 55°C for 30 s and 68°C for 5 min, and a final extension step at 72°C for 7 min. The amplified products were recovered from 2.0% agarose gels, cloned into the pGEM-T vector, and transformed into *Escherichia coli* DH5α. Sequencing was performed commercially and sequence analyses were performed using BLAST from the NCBI (http://www.ncbi.nlm.nih.gov/Tools/).

The expression pattern of α-galactosidase during tartary buckwheat germination

The primers for *FtαGAL* were designed based on the obtained cDNA sequence. The housekeeping gene β-actin was used as internal reference. The primer pairs for *FtαGAL* were FtαGAL-F: 5′-GATACCCTCCCATGCGTGATGC-3′ and FtαGA-R: 5′-GCATAGGCTGCC CACTTGTCAT-3′. The deduced amplification length was 203 bp. The primer pair for β-actin was actin-F: 5′-GCTGGATTTGCTG-GAGATGATGC-3′ and actin-R: 5′-CTTCTCCATGTCA TCCCAGTTGCT-3′ and the deduced amplification length was 196 bp.

Total RNA and first-stand complementary cDNA of buckwheat seeds at different germination stages were prepared as described above. The internal reference gene β-actin and target gene FtαGAL were analyzed in one plate, and each reaction was repeated three times to access the reproducibility. The cycling protocol consisted of denaturation at 95°C for 5 s, annealing at 58°C for 15 s, elongation at 72°C for 20 s, and the PCR reaction was run for 40 cycles. The fluorescence data was collected at 81°C for 20 s. The model $2^{-\Delta\Delta CT}$ for comparing relative expression results between samples in real-time PCR was applied. The expression of target gene, normalized to the reference control and relative to a calibrator (time-zero sample) is given by $R = 2^{-\Delta\Delta CT}$, where ΔΔCT = ΔCT sample − ΔCT control. All samples were performed in triplicates. Positive and negative controls were performed on each plate.

Results

Seed germination of tartary buckwheat

Seed priming enhanced tartary buckwheat seed performance with respect to the speed and uniformity of germination (Fig. 1). The tartary buckwheat seed, after priming, began to germinate just 12 hours after incubation on wet paper layers. In the following time, seed germinated quickly and reach 73.2% at 24th h , then reached the maximum germinability as high as 94% at 36th h. It indicates that the fastest germination must take place between 12 h and 24 h and the complicated physiological and biochemical changes may occur in this period.

The contents of DCI and total fagopyritol during tartary buckwheat germination

The contents of DCI and total fagopyritol in tartary buckwheat seeds were determined at different germination stages (0–72 h; Fig. 2). The DCI contents increased dramatically during the early germination. At 24 h, it reached the maximum level of 33.4 μg/seed, which was about 2.3 times that of time-zero seeds. However, after 24 h, the DCI content in tartary buckwheat sprouts sharply decreased. After 60 h, the level was lower than that of time-zero seeds. Therefore, the optimum time for tartary buckwheat germination was 24 h, yielding the maximum DCI content. The total fagopyritol content revealed an opposite trend, decreased during the tested period from 214.6 (0 h) to 46 μg/seed (72 h). The breakdown of fagopyritol may release D-chiro-inositol and galactose, indicating that they may play a special role during early germination.

Enzyme activity assay of α-galactosidase during the germination

Acid α-galactosidase has great influence on seed development and germination, therefore, recent studies have paid much attention to it [28]. The activities of the alkaline α-galactosidase were very low and changed little during the buckwheat seed germination (data not shown). The activities of acidic α-galactosidase in tartary buckwheat seeds were measured at different germinating periods (Fig. 3). The activity of α-galactosidase exhibited high even at time-zero and increased slowly during the next 36 h of germination

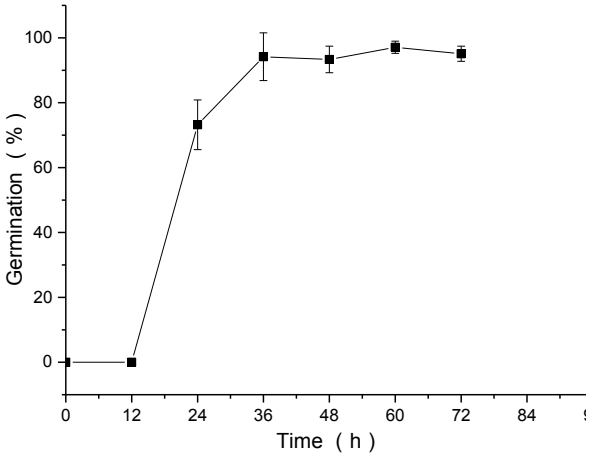

Fig. 1 Time course of germination of primed tartary buckwheat during the first 72 h. Data presented are means ±*SD* (*n* = 3).

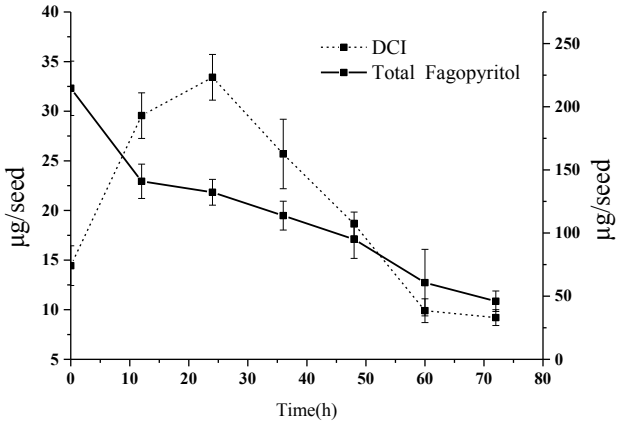

Fig. 2 Changes in DCI and total fagopyritol content of tartary buckwheat in germinating tartary buckwheat. Data presented are means ±*SD* (*n* = 3).

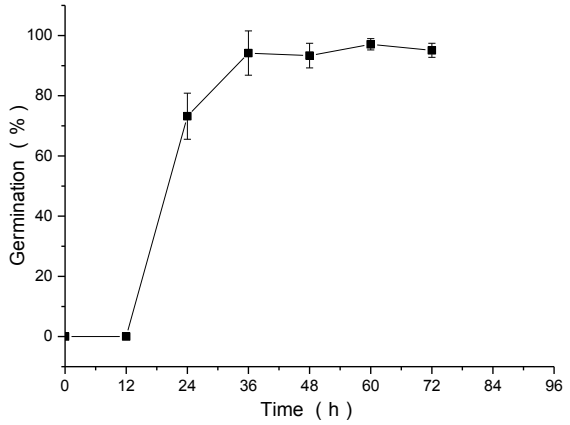

Fig. 3 α-galactosidase activity in primed tartary buckwheat seed during germination. Data presented are means ±*SD* (*n* = 3).

(from 0.30 to 0.36 nkat/seed). However, it decreased rapidly and at the end of the germination it was only 0.12 nkat/seed.

Cloning and identification of *FtaGAL* gene

The consecutive amino acid sequences of α-galactosidase in dicotyledonous were aligned and the protein exhibited a highly conservative sequence (Fig. 4). The degenerate primers were used and the cloned partial cDNA of *FtaGAL* is 293 nucleotides. Multiple alignments revealed a high degree of homology between deduced amino acid sequence of Ftα-GAL and α-Galactosidase of other plants. The highest identity was found to be 93% similarity with *P. vulgaris*. The deduced sequence covered 23% of the full length of the *P. vulgaris* α-galactosidase protein sequence. Ftα-GAL showed less homology with that of *Glycine max* (91%), *Cicer arietinum* (90%) and *Salvia mitiorrhiza* (90%).

Expression of α-galactosidase gene in tartary buckwheat seed during germination

Quantitative PCR was used to analyze the gene expression pattern during tartary buckwheat seed germination (Fig. 5). The mRNA level of α-galactosidase in buckwheat seeds increased steadily during early germination. At 24 h it reached the peak as 2.59 fold in comparison with that of time-zero seeds. Up-regulation of the genes responsible for the fagopyritol breakdown was expected, which was in accordance with the activity of α-galactosidase in tartary buckwheat seed. However the gene expression was found to be down-regulated at the later stages of germination, it declined to only 0.12 fold relative to control at the end of the germination. This correlated with the decrease of the activity of α-galactosidase in buckwheat germination.

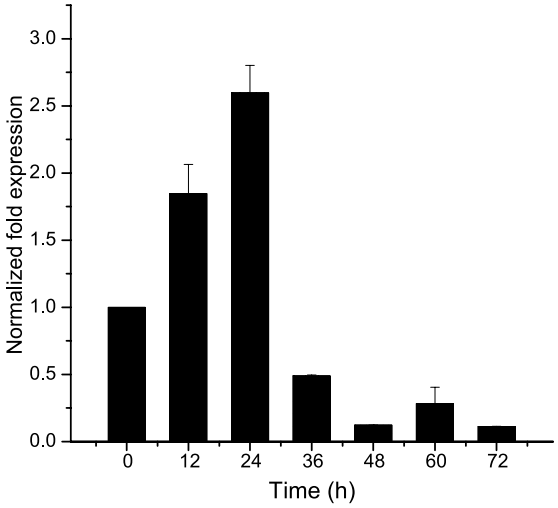

Fig. 5 Expression pattern of α-galactosidase gene during tartary buckwheat germination. The relative expression of α-galactosidase was quantified in comparison with the ACTIN using quantitative PCR with gene-specific primers. The values represent the average of three independent samples. Data presented are means ±*SD* (*n* = 3).

Fig. 4 The consecutive amino acid sequences of α-galactosidase gene in dicotyledon.

Discussion

Buckwheat has attracted increasing attention as a potential functional food for a long time. However, the best usage of it is rarely reported. Elicitor molecules such as salicylic acid (SA) and methyl jasmonate (MeJA) have been used to induce the high yield of free DCI in buckwheat [29]. DCI was significantly higher in the metallic additives treated samples than in the control during the tartary buckwheat germination [30]. The second metabolites have been demonstrated to be accumulated in some plants, by stimulating the activity of enzymes [31]. Germination has been reported to stimulate the production of second metabolites in some cereal seeds such as buckwheat [32]. Seed priming is a promising treatment to improve the rate and uniformity of germination and is associated with increase of enzyme activity in rice seed germination [33]. The seed priming method is used to induce the high yield of DCI during the germination process in this paper and the results show that it reaches the highest level of 33.42 μg/seed at 24 h after imbibition, which provides us the optimum time to make better use of the buckwheat seed for free DCI. These phenomena may attribute to the biochemical metabolism in seed germination [34]. Similarly Zalewski et al. observed that DCI content kept increasing in the first 48 h of seed germination in yellow lupin [35]. In addition, Pathak also found that soaking and early germination converted soybean seeds into an effective blood sugar regulator [36].

Galactosidase play an important role during plant germination. The initial enzymes in RFOs catabolism are α-galactosidases, which hydrolytically remove the terminal galactose moiety of RFOs. During soybean seed germination, the content of raffinose oligosaccharides decrease substantially, while the α-galactosidase activity increases [37]. In our paper we found that the total fagopyritol content decreased while the free DCI content increased during buckwheat germination, which indicated that DCI may be released from the fagopyritol. The analysis between α-galactosidase activities and DCI content revealed a significant positive correlation in germinated tartary buckwheat [30]. We employ the priming method in this paper and the results show that the activity of α-galactosidase exhibited high in time-zero seeds. This may be attribute to the priming treatments significantly mobilize the α-galactosidase activity even before imbibition.

The gene of α-galactosidase have been cloned and analysed in tomato seed, melon fruit and coffee bean [38–40]. Feurtado et al. [38] observed that α-galactosidase gene transcription gradually increased during tomato seed germination by using real-time fluorescence quantitative PCR. In this paper we cloned the partial sequence of α-galactosidase of tartary buckwheat and the gene expression pattern was also evaluated. The results were in close agreement with the enzyme activity of α-galactosidase and profiles of DCI accumulation.

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Authors' contributions
The following declarations about authors' contributions to the research have been made: contributed to the conducting experiments and writing the manuscript: CFJ; contributed to data acquisition: WHH; contributed to the analysis and interpretation of data: ZC; contributed to the conception and design of this paper: HLG.

Competing interests
No competing interests have been declared.

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