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Expression of flavonoid biosynthesis genes vis-à-vis rutin content variation in different growth stages of *Fagopyrum* species

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ABSTRACT

Buckwheat is one of the field crops with the highest concentration of rutin, an important flavonoid of medicinal value. Two species of buckwheat, *Fagopyrum esculentum* and *Fagopyrum tataricum*, are the major sources of rutin. Seeds of latter contain 40–50× higher rutin compared to the former. The physiological and molecular bases of rutin content variation between *Fagopyrum* species are not known. The current study investigated the differences in rutin content in seeds and in other tissues and growth stages of two *Fagopyrum* species, and also correlated those differences with the expression of flavonoid pathway genes. The analysis of rutin content dynamics at different growth stages, S1–S9 (from seed germination to mature seed formation) of *Fagopyrum* species revealed that rutin content was higher during seedling stages of *F. tataricum* (3.5 to 4.6-fold) compared to *F. esculentum* and then increased exponentially from stages S3 to S6 (different leaf maturing stages and inflorescence) of *F. esculentum*, whereas it fluctuated in *F. tataricum*. The rutin content was highest in the inflorescence stage (S6) of both species, with a relatively higher biosynthesis and accumulation during post-flowering stages of *F. tataricum* compared to *F. esculentum*. The expression of flavonoid pathway genes, through qRT-PCR, in different growth stages vis-à-vis rutin content variation showed differential expression for four genes, PAL, CHS, CHI and FLS with the amounts of transcripts relatively higher in *F. tataricum* compared to *F. esculentum*, thereby, correlating these genes with the biosynthesis and accumulation of rutin. The expression of PAL was highest, 7.69 and 8.96-fold in Stages 2 (seedling stage) and 9 (fully developed seeds) of *F. tataricum* compared to *F. esculentum*, respectively. The expression of the CHS gene correlated with the rutin content because it was highest in the flowers (S6) and fully developed seeds (S9) of both *Fagopyrum* species, with relatively higher transcript amounts (2.13 and 3.19-fold, respectively) in *F. tataricum* (IC-329457) compared to *F. esculentum* (IC-540858). This study provides useful information on molecular and physiological dynamics of rutin biosynthesis and accumulation in *Fagopyrum* species and the correlation of expression of flavonoid biosynthesis genes with the rutin content can be useful in planning for genetic improvement.

Introduction

Flavonoids are the largest group of secondary metabolites involved in many biological functions in plants. Flavonoids are categorized into flavonols, flavones, flavanones, catechins, anthocyanidins and chalcones (Winkel-Shirley, 2001). One of the bioactive flavonoids, rutin, is present in substantial amounts in various plants (McGregor and McKillican, 1952; Attanassova and Bagdassarian, 2009). Rutin has desirable physiological and biological properties, such as anti-oxidation, anti-inflammation, anti-hypertension, vasoconstrictive, spasmolitic and a positive inotropic effect (Campbell, 1997; La Casa et al., 2000; Kuntić et al., 2011; Landberg et al., 2011). Rutin also provides protection against gastric lesions (La Casa et al., 2000), improves sight and hearing (Campbell, 1997), protects against UV light (Gaberscik et al., 2002), lowers plasma cholesterol (Kayashita et al., 1997), protects from oxidative stress (Gong et al., 2010), causes muscle hypertrophy (Kayashita et al., 1997), and also suppresses gallstone formation and cholesterol level (Kuntić et al., 2011).

Buckwheat (*Fagopyrum* sp.), a pseudocereal used for both grains and greens and has a nutraceutical value rich in carbohydrates, vitamins and flavonoids. Rutin is not found in other grains such as wheat, rice, and corn, but is found in higher quantities in buckwheat, which is thus considered to be a major dietary source of rutin. Out of 14 species of *Fagopyrum*, two viz. *Fagopyrum tataricum* (Tartary buckwheat) and *Fagopyrum esculentum* (common buckwheat) are cultivated types and all others occur as wild or escape in the cultivated fields (Rana, 2004). Rutin has been identified in

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higher amounts in *F. cymosum* (0.01%) *F. tataricum* (0.8–1.8%), and *F. esculentum* (0.01%). Tartary buckwheat is less common and often referred to as bitter buckwheat; the bitterness of tartary buckwheat makes this species less suitable for food consumption (Campbell, 1997). The seeds of tartary buckwheat contain higher (40–50×) rutin content (0.8–1.8% d.w.) than that of common buckwheat (0.01% d.w.) (Fabjan et al., 2003; Chauhan et al., 2010; Park et al., 2011). Rutin content was reported to be maximal in flowers of buckwheat (Wagenbreth et al., 1996). The flavonoid content variation has been reported in other plants, such as in barrenwort, potentilla, peanuts and citrus (Ghafar et al., 2010; Tomczyk et al., 2010; Quan et al., 2010).

The biosynthesis and accumulation of rutin and other flavonoids is controlled at the molecular level by the structural and regulatory genes in different plant species (Winkel-Shirley, 2001; Buer et al., 2010). The rutin biosynthetic pathway is a branch of a large phenylpropanoid pathway, and the pathway genes are conserved among different plants and have been characterized at the genetic, biochemical and enzymatic levels in plant species including *Zea mays*, snapdragon, *Petunia*, *Arabidopsis* (Mol et al., 1998; Winkel-Shirley, 2001).

At present, the demand for buckwheat is increasing in the food, pharmaceutical and cosmetic industries due to its nutritional and medicinal value based on the favorable composition of the protein complex with a high content of lysine, fibrous material, mineral compounds, vitamins and rich source of bioflavonoid rutin (Keli, 1991). However, the higher amounts of rutin in inflorescence and mature seeds restrict extraction and commercial production of rutin because both of these growth stages constitute a small fraction of the total biomass of buckwheat. The physiological and molecular basis of higher rutin content in *F. tataricum* is not thoroughly understood. The expression status of anthocyanin biosynthetic genes was investigated independently in *F. tataricum* and *F. esculentum* in leaves, stems, and flowers, showing that these genes were highly expressed in the stems and roots (Li et al., 2010; Park et al., 2011). The physiological and molecular dissection of higher rutin content in *F. tataricum* and its correlation with the expression of flavonoid pathway genes would provide avenues for genetic improvement of buckwheat for enhanced rutin production.

We report here the biosynthesis and accumulation of rutin in different growth stages (seed germination to seed maturation) of two *Fagopyrum* species, *F. tataricum* (IC-14889; tartary buckwheat and IC-329457; rice tartary buckwheat) and *F. esculentum* (IC-540858; common buckwheat) along with the expression of flavonoid biosynthesis pathway genes through qRT PCR analysis.

Materials and methods

Plant material and growth conditions

The seeds of *Fagopyrum* sp. were procured from the National Bureau of Plant Genetic Resources (NBPGR), Regional Research Station, Phagli, Shimla (H.P.), India. For rutin content analysis and expression analysis of flavonoid pathway genes, seeds of three accessions, IC-14889 (*F. tataricum*; Tartary buckwheat), IC-329457 (*F. tataricum*; Rice Tartary buckwheat) and IC-540858 (*F. esculentum*; Common buckwheat) were germinated in a potting mixture consisting of soil and vermiculite in a ratio of 1:1. The seedlings were grown from germination to mature seed formation under controlled conditions of light (intensity 300–1400 Lux, i.e., 4.05–18.9 $\mu mol\, m^{-2}\, s^{-1}$), temperature ($25 \pm 2\,^{\circ}C$), humidity (≈70%), and photoperiod of 14 h day/10 h night. Samples of different growth stages (Fig. 1) were harvested between 9 and 10 AM (June to September), immediately frozen in liquid nitrogen and stored at −80 °C for further analysis in the quantification of rutin, isolation of genomic DNA and total RNA.

Estimation of rutin by HPLC

Preparation of extract

Before extraction, the seeds, flowers, leaves and tissues of different growth stages were powdered in liquid nitrogen by using pestle and mortar. The 100 mg of tissue powder was extracted in 80% methanol, incubated for 2 h at 60 °C, centrifuged and filtered through 22 μm filters for further analysis by HPLC to estimate rutin content.

Quantification of rutin

The extract in 80% methanol was analyzed with a Waters 515 HPLC system equipped with a model 515 solvent pump, ASI-100 autosampler (USA), a PDA-Waters 2996 photodiode array detector, Waters In-Line degasser AF and Empower pro Software. A Phenomenex luna® C_{18}100 with a reversed-phase column (250 mm × 2.6 mm, 3 μm) was used. Flavonoids were detected at 350 nm. The mobile phase was acetonitrile and 0.04% TFA in a linear gradient for 35 min at 30 °C at a flow rate of 0.9 ml/min and an injection volume of 20 μl. Flavonoids were identified by comparison of their retention times with those of pure standards and quantified individually, based on standard curves of rutin (Sigma; Fig. 2). Rutin quantification was performed with the linear calibration curves of standard compounds. The significant differences between rutin contents were statistically analyzed.

Isolation of genomic DNA and total RNA

Genomic DNA was isolated from leaves of *Fagopyrum* species following the protocol of Murray and Thompson (1980). Total RNA was isolated from *Fagopyrum* samples of different growth stages by using Raflex RNA isolation kit (GeNei™) by following manufacturer's instructions. The quality of DNA and RNA was checked by 1% (w/v) ethidium bromide-stained agarose gel and through the absorbance spectrum at wavelengths 260 nm and 280 nm.

Cloning and sequencing of flavonoid (rutin) biosynthesis genes in buckwheat

The nucleotide and protein sequences of genes were retrieved from different plant species in Genbank (http://www.ncbi.nlm.nih.gov/genbank/) and the multiple sequence alignments (MSA) were done so as to identify conserved sequence regions. Though the extent of sequence similarity was low in coding regions of flavonoid biosynthesis genes, short patches of conserved sequences were identified. Primer pairs were designed from the conserved regions of gene sequences (Supplementary Table 1) and tested on genomic DNA and cDNA of *Fagopyrum* species.

For amplification, the PCR was performed on 30 ng genomic DNA and cDNA separately with varying amount of primer pairs, Mg^{2+}, dNTPs and *Taq* DNA polymerase. Amplification programs included 94 °C for 3 min, 30 cycles of 94 °C for 30 s, annealing temperature (63–55 °C) for 45 s, 72 °C for 2 min and a final extension of 7 min at 72 °C. 10 μl of each PCR product was mixed with 2 μl of 6× gel loading dye (0.2% bromophenol blue, 0.2% xylene cyanol dye and 30% glycerol) and electrophoresed in a 2% agarose gel prepared in 0.5× Tris borate–EDTA (TBE) buffer. The gels were analyzed using the gel documentation system AlphaImager EP (Alpha Innotech Corp., USA). The PCR products were cloned in pGEMT vector (Promega) and sequenced. The identities of sequence similarities