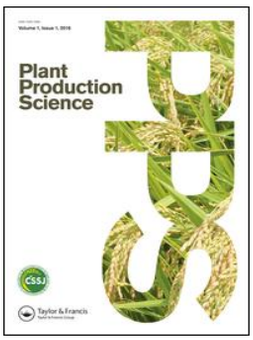

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Changes in seed growth, levels and distribution of flavonoids during tartary buckwheat seed development

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ABSTRACT

We investigated the change in seed shape and weight as well as the accumulation and distribution of anthocyanins, melanin, and flavonoids during tartary buckwheat seed development. Pericarp dry weight increased slowly while the increase in flour dry weight was much greater. Increases in the dry weight of the embryo and endosperm were associated with change in seed shape. The highest anthocyanin content was observed in stage two while the highest melanin content occurred in stage five. There was a considerable decrease in total flavonoids, rutin, quercetin and kaempferol in mature seeds relative to seeds that were still developing. Additionally, there were significant correlations (correlation coefficient > .900, $p < .05$) among the changes in rutin, quercetin and kaempferol between flour and the pericarp. In mature seeds, 95% of the flavonoids were present in flour.

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Tartary buckwheat; seed development; flavonoids; anthocyanin; melanin; pericarp

CLASSIFICATION
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Introduction

Tartary buckwheat [*Fagopyrum tataricum* (L.) Gaertn] is a dicotyledonous plant belonging to the family *Polygonaceae*. It is well known for its nutritive and medicinal value because of its abundance of amino acids, dietary fibre, mineral compounds and phenolic compounds (Bonafaccia et al., 2003; Qin et al., 2013; Wang et al., 2013). Tartary buckwheat also contains large amounts of flavonoid compounds including rutin, quercetin and flavone C-glycosides (Zielińska et al., 2012). Many studies have reported that phenolic compounds can decrease high blood pressure and lipid levels by decreasing the absorption of dietary cholesterol and lowering plasma cholesterol levels (Kim et al., 2009; Lee et al., 2000). Tartary buckwheat contents have also exhibited antioxidant and lipid peroxidation activities (Guo et al., 2011; Liu et al., 2007, 2008). Because of its potential health benefits, Tartary buckwheat is being consumed by an increasing number of people globally (Li & Zhang, 2001).

Tartary buckwheat seeds are an important resource that can be processed into various food products such as tea, noodles and pastries (Ma et al., 2013; Merendino et al., 2014; Qin et al., 2013). As seeds mature, they undergo changes in terms of shape, colour and dry weight (DW). Anthocyanins in plants provide colour to fruits, leaves and flowers (Harborne, 1963). Researchers have demonstrated that different anthocyanins accumulate during the various growth stages of buckwheat flowers (Suzuki et al., 2007). Additionally, the levels of anthocyanin present in tartary buckwheat sprouts was affected by exposure to specific light/dark cycles (Peng et al., 2015). However, to the best of our knowledge, no studies have focused on the changes in anthocyanin content and seed shape during different growth stages of tartary buckwheat seeds. Moreover, flavonoids are the primary active components that accumulate during tartary buckwheat growth (Gupta et al., 2011). However, it is unclear how flavonoids such as rutin, quercetin and kaempferol change and localise during seed development. Because the seed is the most important source of beneficial tartary buckwheat production, a more complete characterisation of the flavonoid changes occurring in seeds may have implications for improving production quality in choosing the material and processing. Additionally, the results of this study may help researchers develop treatments to improve the flavonoid content of seeds.

Materials and methods

Plant material

Tartary buckwheat cultivar 'Xiqiao 1' was provided by Xichang College (Sichuan, China) and planted at Chengdu

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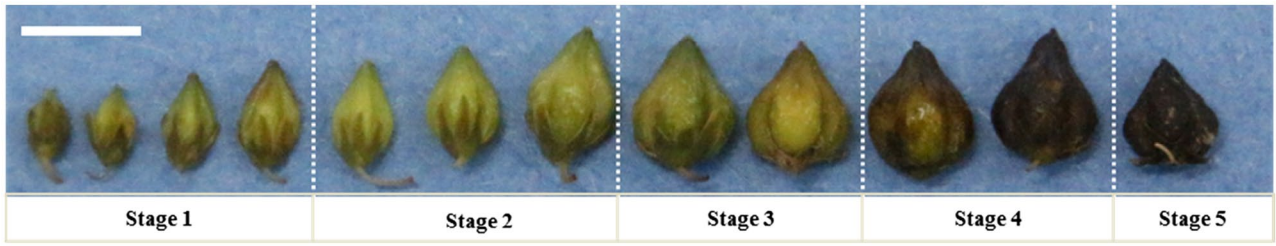

Figure 1. Different developmental stages of tartary buckwheat seeds. Bar = 0.5 cm; Stage 1: Seed formation started; Stage 2: Filling stage, the endosperm is liquid or milky; Stage 3: Milk-ripe stage, the endosperm is solidifying; Stage 4: The pericarp is changing green into black; Stage 5: The seed matured, and pericarp was totally black.

University (30°65′N and 104°19′E, 495 m altitude), Sichuan Province, China. The seeds were sown on 5 September 2014, and harvested on 20 December 2014. During the season, the mean temperature is 20.7 °C, and the total precipitation is 60.7 mm. Seedlings were thinned to the final density (7×10^5 ha^{-1} plants) 15d after germination. The soil of the field is sandy loam in texture and acidic (pH 6.59) with 128.0, 23.7 and 116 mg kg^{-1} available N, P and K, respectively; 1.29, 0.87 and 14.8 g kg^{-1} total N, P and K, respectively; and 22.2 g kg^{-1} organic matter. Soil tests were done on samples taken from the upper 20 cm of the soil. The synthetic fertilizer (N: P: K = 15:15:15) was applied as basal fertilizer at the rate of 300 kg ha^{-1}. Tartary buckwheat seeds were collected at different developmental stages (Figure 1) and dried at 105 °C for 30 min and then at 60 °C to obtain a constant weight. The final samples included whole seeds, the pericarp and flour (the hulled seeds). The weight of 20 seeds was used in calculations of accumulation in weight and the distribution of rutin, quercetin and kaempferol in organs. The collected seed parts were frozen in liquid nitrogen and stored at –80 °C for subsequent analyses of anthocyanin and melanin content.

Analyses of anthocyanin and melanin content

The relative anthocyanin content was analysed based on an established procedure (Pirie & Mullins, 1976). The pericarp was separated from frozen seeds and ground in the presence of liquid nitrogen. About 5 mL acetone was added to the ground material at 30 °C until the pericarp was no longer green. The sample was centrifuged at 1500 × *g* for 5 min and the supernatant discarded. The sediment was extracted with 5 mL 5% HCl–methyl alcohol at 60 °C for 1 h and centrifuged. The extraction procedure was repeated twice. The absorbance of the final supernatant at 530 and 600 nm was determined with an UV 3200S ultraviolet spectrophotometer (MAPADA, Shanghai, China). Finally, relative anthocyanin content was calculated according to the formula: concentration = $[(A_{530} - A_{600}) / 0.1]$ g^{-1} fresh weight (FW).

Melanin was extracted from the sediment by the addition of 2 mL 2% NaOH at 70 °C for 30 min. This step was repeated until the colour disappeared. The sample was centrifuged and the absorbance of the supernatant at 290 nm was determined. Relative melanin content was recorded as A_{290} g^{-1} FW (Ye et al., 2001).

Flavonoid extraction from tartary buckwheat

The whole seeds, pericarp and flour of differential stages were ground to a fine powder using a pestle and mortar. Tissue powder (0.1 g) was extracted with 15 mL 70% methanol and incubated for 2 h at 60 °C with oscillating shaking at 200 rpm. After centrifugation and filtering with Medium-speed qualitative filter paper, the extract was filled up to 25 mL with 70% methanol for subsequent analysis.

Quantitative analysis of total flavonoids

Total flavonoid content was determined based on an established procedure involving spectrophotometry with aluminium chloride (Chang et al., 2002). Briefly, 1 mL extract was mixed with 2 mL 0.1 M aluminium chloride and 3 mL 1 M potassium acetate. To the mixture, 4 mL 70% methanol was added for a final volume of 10 mL. The mixture was mixed and its absorbance at 420 nm was measured after 30 min. Total flavonoid content was calculated with a calibration curve (R^2 = .999) for rutin and expressed as rutin equivalent (mg) mg^{-1} DW.

Quantitative analysis of rutin, quercetin and kaempferol by high-performance liquid chromatography

External standards for rutin, quercetin and kaempferol were purchased from Herbpurify Co., Ltd. (Chengdu, China). Extracts in 70% methanol were filtered through a 0.45 µm filter for high-performance liquid chromatography (HPLC) analysis using the Shimadzu LC-10AT VP Plus (Kyoto, Japan). The analysis was monitored at 320 nm

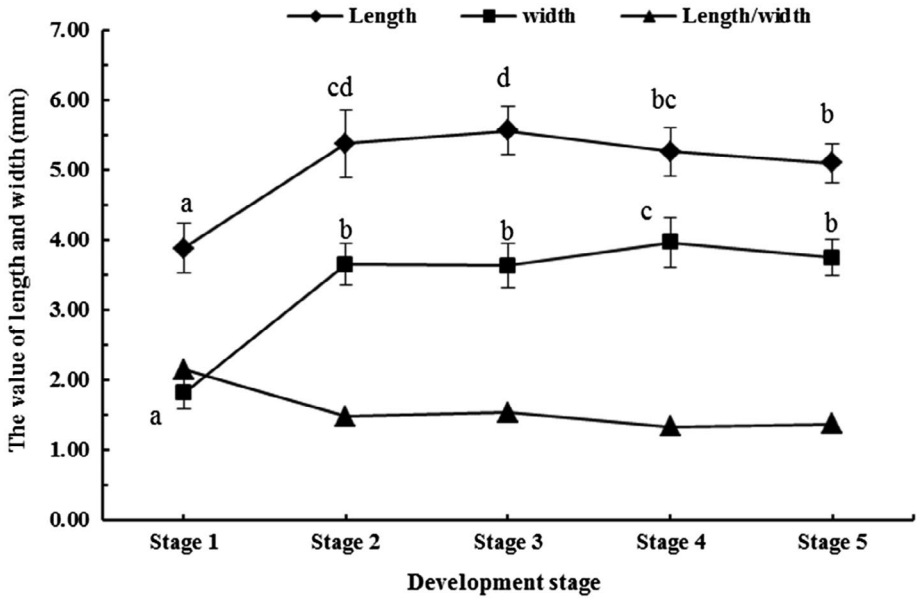

Figure 2. Changes in seed shape during seed development.
Notes. Data are presented as the mean ± SD. Seeds were divided into different developmental stages with 15 seeds measured at each stage. Different letters denote significant differences ($p < 0.05$).

and completed using a Kromasil 100–5C C_{18} column (250 mm × 4.6 mm, 5 µm). Two mobile phases were prepared for mixing. The ratios of methanol: water: acetic acid for mobile phases A and B were (v/v/v) 5: 92.5: 2.5 and 95: 2.5: 5, respectively. The column was maintained at 35 °C and the flow rate was set at 1.0 mL min^{-1}. The injection volume was 20 µL. Flavonoids were identified by comparing their retention times with those of standard compounds, and the peak area was calculated to assess the content of rutin, quercetin and kaempferol (mg g^{-1} DW).

Statistical analyses

All results presented in the figures and tables were calculated from three replicates and are expressed as the mean ± standard deviation (SD). Significant differences between developmental stages were determined by the Student's *t*-test with SPSS, version 17.0 (SPSS Inc., Chicago, IL, USA). Correlation analyses were performed with Pearson's correlation test.

Results

Changes in seed shape during seed development

During seed development, the seed shape changed considerably (Figure 2). Seed length and width increased significantly ($p < .05$) from stages one to two. The length increased from 3.88 to 5.37 mm while the width increased from 1.81 to 3.65 mm. The ratio of seed length to width after stage three indicated that seeds became conical. The change in seed shape might be due to the development of the embryo and endosperm (Stevens, 1912).

Changes in pericarp colour during seed development

Anthocyanin and melanin content of pericarp varied significantly ($p < .05$) during tartary buckwheat seed development (Figure 3). Anthocyanin content was highest during stages two and three, while maximal melanin content was observed in the mature seeds of stage five. The ratios of anthocyanin to melanin were 0.26, 0.59, 0.44, 0.14, and 0.04 in stages one to five, respectively, which corresponded to the colour changes indicated in Figure 1.

Changes in growth during seed development

The weight of seeds, flour, and the pericarp changed significantly ($p < .05$) during seed development (Figure 4). Seed FW significantly ($p < .05$) increased and was the highest in stage four (0.68 g/20 seeds). Similarly, seed DW and flour DW significantly increased, but were highest in stage five, 0.40 and 0.32 g/20 seeds, respectively. Because the pericarp only forms part of the seed, its abundance was less than that of flour. The pericarp DW did not significantly ($p > .05$) increase after stage four. Total moisture content was affected by the increasing flour and pericarp DWs, with the highest moisture content observed in stage one seeds (83.7%). This was twofold higher than the moisture content observed in stage five.

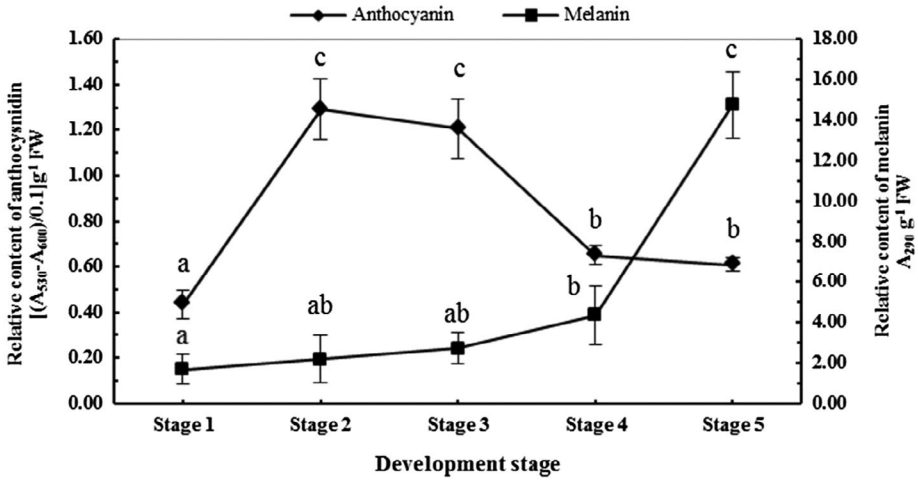

Figure 3. Changes in anthocyanidin and melanin content during seed development.
Notes. Data are presented as the mean ± SD. Seeds were divided into different developmental stages. Each value is the mean of three replicates. Different letters denote significant differences ($p < 0.05$).

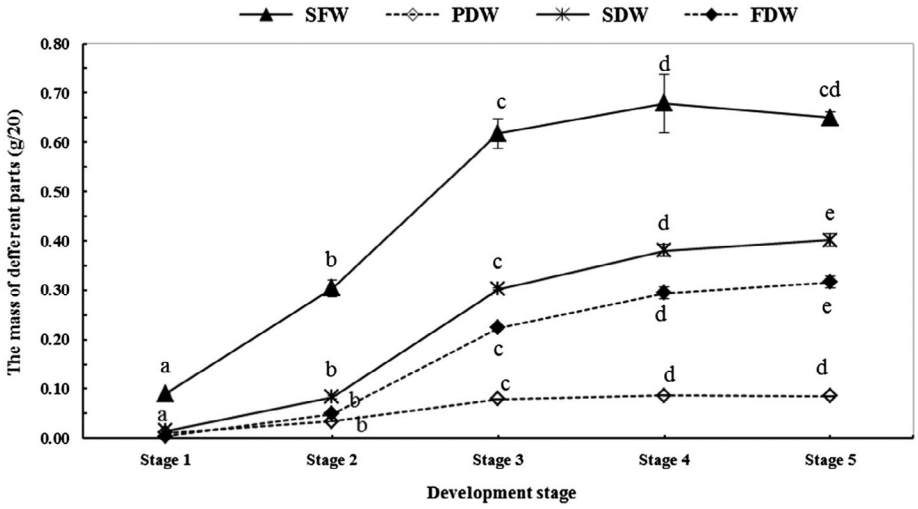

Figure 4. Changes in fresh and DW of seed components during seed development.
Notes. Data are presented as the mean ± SD. Seeds were divided into different developmental stages. Each value is the mean of three replicates. Different letters denote significant differences ($p < 0.05$). SFW, seed fresh weight; PDW, pericarp dry weight; SDW, seed dry weight; FDW, flour dry weight.

Total flavonoid content during seed development

Total flavonoid content for the five seed development stages is presented in Figure 5. Flavonoid content was highest in stage one for whole seeds, flour and the pericarp with values of 36.70, 60.28 and 28.74 mg g^{-1} DW, respectively. The total flavonoid content was lowest in the pericarp of mature seeds (i.e. stage five) with only 2.96 mg g^{-1} DW. The flavonoid content of whole seeds and flour in the same stage were 16.79 and 19.23 mg g^{-1}, respectively.

Rutin content during seed development

Rutin content was assessed using HPLC by comparing its retention times with those of a standard compound (Figure 6(a), peak 1). The results are presented in Figure 7. Rutin concentration was highest in flour in stage one, with a value of 28.57 mg g^{-1} DW. When seeds matured, whole seed rutin content decreased significantly ($p < .05$) to 5.58 mg g^{-1} DW. Rutin did not accumulate in the pericarp, which is inedible. In fact, there was a sharp decrease in pericarp rutin content from 13.96 to 0.37 mg g^{-1} DW from stage one to five.

Quercetin content during seed development

Quercetin content results are presented in Figure 8. Similar to the results for total flavonoids and rutin, quercetin content in flour was higher than that in the pericarp in all

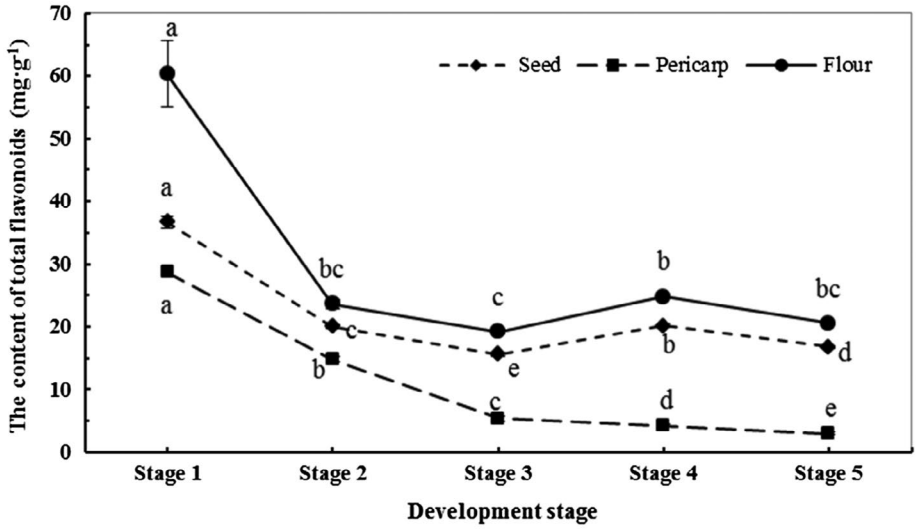

Figure 5. Changes in total flavonoid content during seed development.
Notes. Data are presented as the mean ± SD. Seeds were divided into different developmental stages. Each value is the mean of three replicates. Different letters denote significant differences ($p < 0.05$).

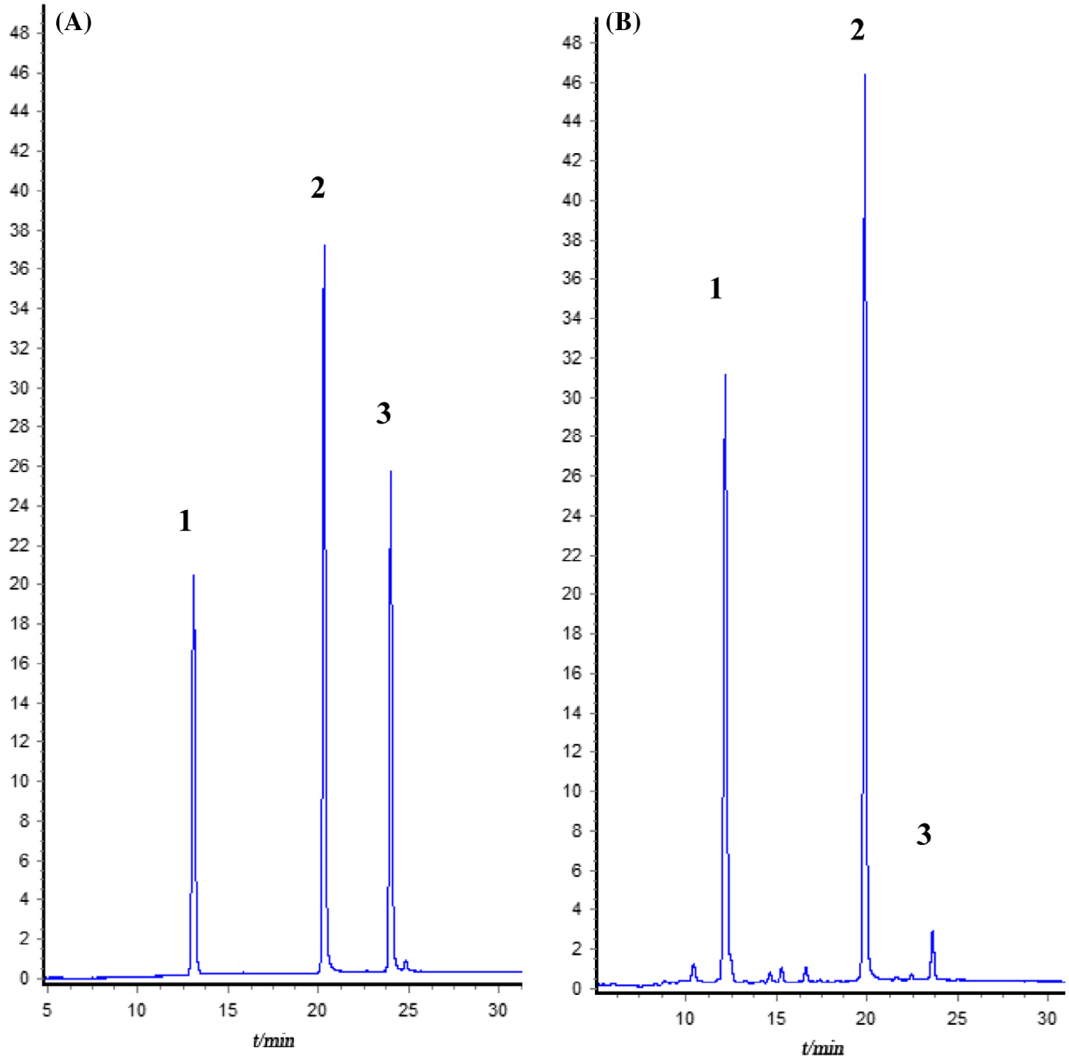

Figure 6. HPLC map of a reference substance (A) and tartary buckwheat sample (B). Rutin, quercetin, and kaempferol were identified by comparing their retention times with those of standard compounds. Peak area was calculated to assess the content of rutin (1), quercetin (2), and kaempferol (3).

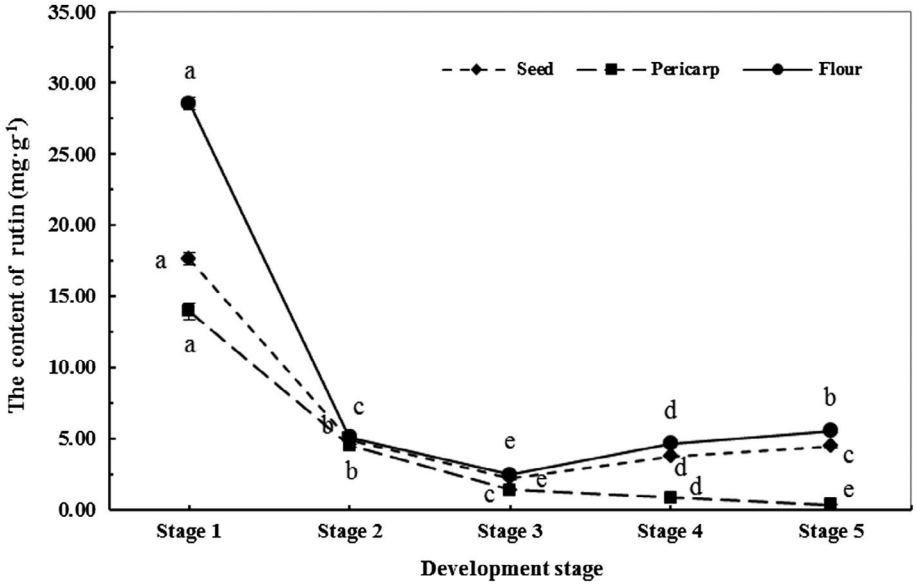

Figure 7. Changes in rutin content during seed development.
Notes. Data are presented as the mean ± SD. Seeds were divided into different developmental stages. Each value is the mean of three replicates. Different letters denote significant differences ($p < 0.05$).

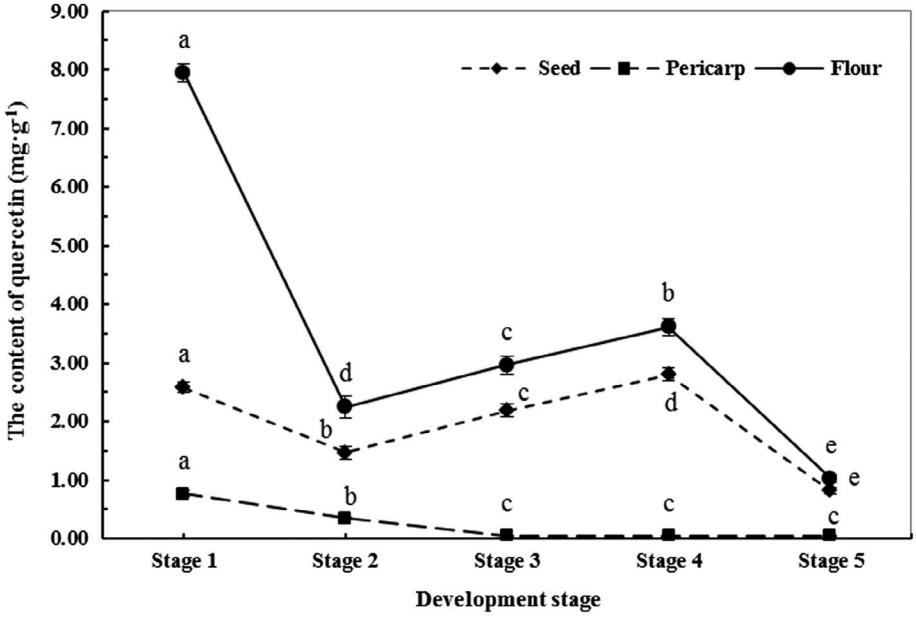

Figure 8. Changes in quercetin content during seed development.
Notes. Data are presented as the mean ± SD. Seeds were divided into different developmental stages. Each value is the mean of three replicates. Different letters denote significant differences ($p < 0.05$).

seed development stages. Quercetin content was highest in stage one, with values of 7.95 mg g^{-1} DW in flour and 0.77 mg g^{-1} DW in the pericarp. Interestingly, the total quercetin content in stage one was approximately double that of stage four and about three and a half times higher than that of stage five. This suggests that the seeds of stage four may be a better source of nutritious food products than the mature seeds of stage five. As shown in Figure 8, the pericarp quercetin content continued to decrease from stage one to five. In stage five, the quercetin content of the pericarp was 95% lower than that of flour (1.03 mg g^{-1} DW)

Kaempferol content during seed development

The changes in kaempferol content during seed development (Figure 9) were consistent with those of quercetin (Figure 8), except for the minor differences between flour and the pericarp in stage one. The highest and the

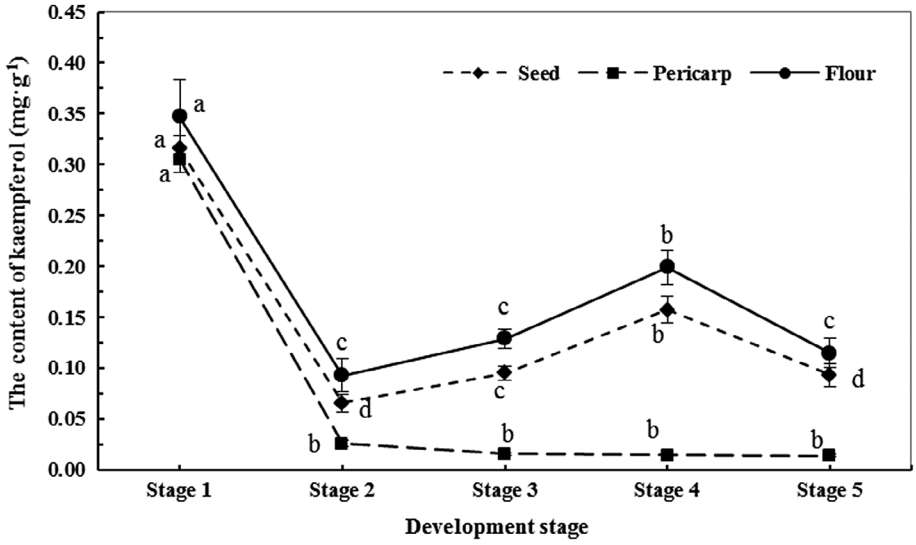

Figure 9. Changes in kaempferol content during seed development.
Notes. Data are presented as the mean ± SD. Seeds were divided into different developmental stages. Each value is the mean of three replicates. Different letters denote significant differences ($p < 0.05$).

Table 1. Analysis of the correlations among several components in flour and the pericarp during seed development.

		Pericarp			Flour		
		Rutin	Quercetin	Kaempferol	Rutin	Quercetin	Kaempferol
Pericarp	Rutin	1					
	Quercetin	0.988**	1				
	Kaempferol	0.968**	0.926*	1			
Flour	Rutin	0.955*	0.920*	0.994**	1		
	Quercetin	0.895*	0.828 ns	0.930*	0.904*	1	
	Kaempferol	0.823 ns	0.746 ns	0.914*	0.912*	0.960**	1

Notes. Correlations were assessed using SPSS 17.0 and the correlation coefficients are presented; ns, no significant difference ($p > .05$).
*significant difference at $p < .05$;
**significant difference at $p < .01$.

maximum flour kaempferol content was observed in stages one and four, with values of 0.35 and 0.20 mg g^{-1} DW, respectively (Figure 9). There was a sharp decrease in pericarp kaempferol content from stage one to two and no significant accumulation during stages two to five. The pericarp kaempferol content in stage one (0.31 mg g^{-1} DW) was almost 20-fold higher than that in stage five.

Correlation among flavonoids during seed development

The correlations among the contents of rutin, quercetin and kaempferol during seed development were analysed (Table 1). A significant correlation was observed among rutin, quercetin and kaempferol in flour and the pericarp. In the pericarp, there were significant correlations between rutin and quercetin, rutin and kaempferol, quercetin and kaempferol, with correlation coefficients of 0.988 ($p < .01$) and 0.968 ($p < .01$) and 0.962 ($p < .05$), respectively. In flour, the significant correlation was observed 0.904 ($p < .05$), 0.912 ($p < .05$) and 0.960 ($p < .01$), respectively, as well as in pericarp.

Flavonoid distribution during seed development

The results of our investigation of flavonoid distribution in flour and the pericarp are presented in Figure 10. Total flavonoids, rutin and kaempferol were present more in the pericarp than in flour in stage one. The proportions of total flavonoids, rutin and kaempferol in the pericarp in stage one were 58.6, 59.1 and 72.3%, respectively. However, only 22.4% of quercetin was observed in the pericarp in stage one. For all components, the common trend was that the pericarp proportion rapidly decreased as the seeds developed. In stage five, flour contained more than 95% of the flavonoids, essentially decreasing the nutritional or medicinal value of the pericarp.

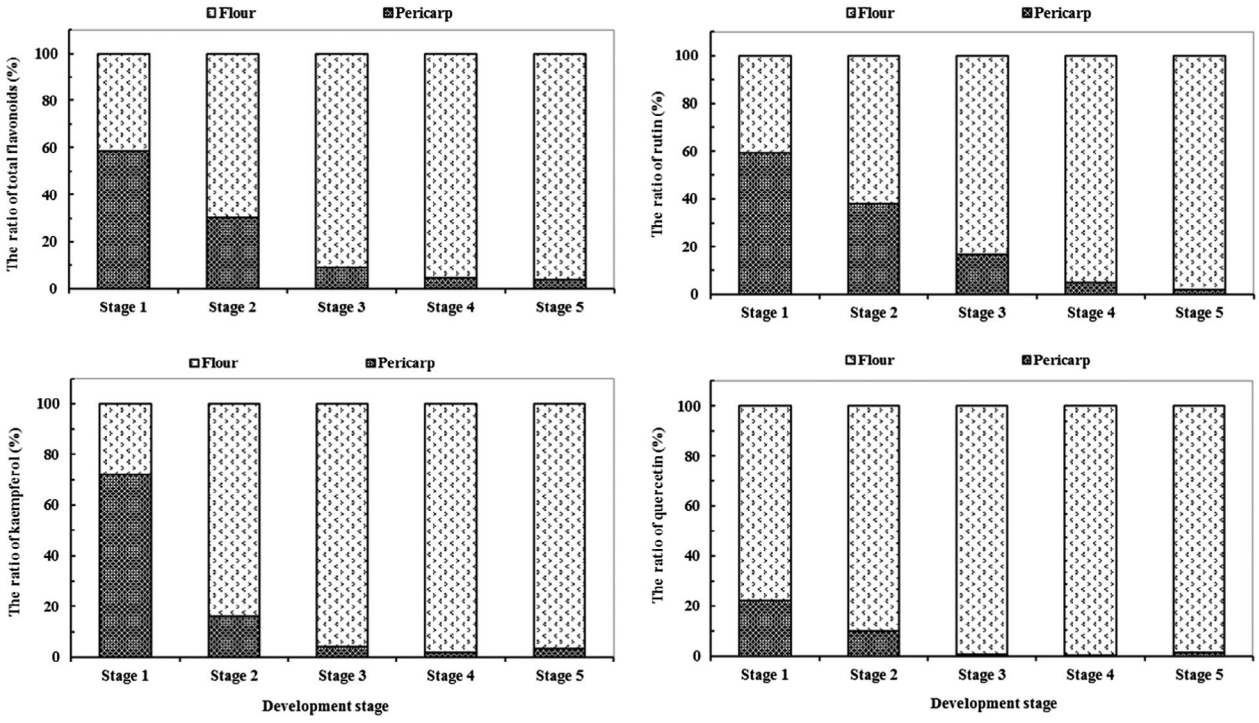

Figure 10. Changes in the distribution of total flavonoids, rutin, quercetin and kaempferol during seed development.

Discussion

In this study, we described the seed morphological changes that occurred during development. The pericarp wraps around the embryo and endosperm, and expanding pericarp tissues increase the seed DW. The rapid changes in the pericarp provided an enough interspace to accumulate for the embryo and endosperm during growth (Stevens, 1912). Additionally, the embryo and endosperm DW continually increased during seed development (Figures 2 and 4, flour dry weight (FDW)).

Anthocyanins, which are flavonoids, have been detected in Tartary buckwheat roots, leaves, stems, flowers, and shoots (Park et al., 2011; Peng et al., 2015). The antioxidant potency of anthocyanins is concentration-dependent (Gabrielska et al., 1999; Watanabe, 2007). In this study, we determined the anthocyanin content in the pericarp at different seed development stages. The relative anthocyanin content was highest in stages two and three (Figure 3). Additionally, flavonoids such as quercetin and kaempferol were abundant in flour (Figures 5, 8, and 9). These results provide evidence that young seeds might represent an important source of nutritious plant material with medicinal value. We also detected a significantly increase ($p < .05$) in melanin of pericarp between stages four and five. And melanin is synthesised by a series of chemical reactions and regulated by the oxidation of tyrosine by tyrosinase (Eisenman & Casadevall, 2012). An increase in melanin abundance in pericarp may lead to protective effects to seed because the reactive quinone intermediates in the melanin biosynthetic pathway exhibit antibiotic properties and the polymer is important in strengthening plant cell walls (Riley, 1997).

We examined flavonoid accumulation and distribution using precise developmental stages. The results regarding DW increases were consistent with those of previous studies (Gupta et al., 2011; Li et al., 2012; Zielińska et al., 2012). The highest total flavonoid, rutin, quercetin and kaempferol contents in flour and the pericarp were observed in stage one. This may be because young seeds develop from flowers, which have high flavonoid content (Park et al., 2004; Wagenbreth et al., 1996). During seed development, the total flavonoid, rutin, quercetin and kaempferol contents of flour in stage one were 2.93, 5.1, 7.7 and 3.0 times higher than those of stage five while the values for the pericarp in stage one were 9.7, 37.3, 15.3 and 22.1 times higher than those of stage five, and these results indicated the accumulation of flavonoids in flour were higher than in pericarp. Because of the rapid increase in flour and pericarp DW, the considerable decrease in flavonoid content observed in stage two, and it also may be related to the effects of four flavonoid pathway genes, phenylalanine ammonium lyase, chalocone synthase, chalcone isomerase and flavonol synthase, which are positive correlation with the rutin content variation at different growth stages of *Fagopyrum* species. (Li et al., 2010, 2012). The significant correlations among flavonoids observed in our study (correlation coefficient > .900, $p < .05$) suggest the rutin, quercetin and

kaempferol contents are synchronously changing. Thus, improving or increasing flavonoid content may be possible using different treatments (e.g. fertilizers) or exposure to abiotic stresses (e.g. drought and salinity) (Li et al., 2013; Xiang et al., 2013; Zhao et al., 2013). Flour mainly consists of the embryo and endosperm, both of which can accumulate flavonoids during seed development. Research has shown that the embryo has more flavonoids than other tissues (Steadman et al., 2001). This may explain why most of the flavonoids were present in flour during the later developmental stage (i.e. stages four and five). The most notable finding in this study was that the small increases in flavonoid content in stage four. The yield of flavonoid, rutin, quercetin and kaempferol in flour of 20 seeds was 7.32, 1.37, 1.06 and 0.06 mg in stage four, and 6.49, 1.76, 0.33 and 0.04 mg in stage five, respectively, indicate that stage four seeds may be more beneficial in terms of nutrition and medicinal value than the mature seeds in stage five.

Conclusions

Seed development is a complex process involving morphological, physiological and biochemical changes. The increase in embryo and endosperm DW was associated with the changes in seed shape. Anthocyanins behaved differently than the other flavonoids (i.e. total flavonoids, rutin, quercetin and kaempferol). It may be possible to use this difference to select seeds that are suitable for particular beneficial products.

Disclosure statement

No potential conflict of interest was reported by the authors.

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