

Biosynthesis and Accumulation of Flavonoids in *Fagopyrum* spp.

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ABSTRACT

Buckwheat is a multipurpose crop used for both grains and greens and known to have several medicinal and nutritional properties. Buckwheat contains flavonoids such as rutin, anthocyanins, catechins, chlorogenic acid, 4-hydroxy-3-methoxy benzoic acid, caffeic acid, epicatechins, p-coumaric acid, ferulic acid etc. *Fagopyrum esculentum* and *Fagopyrum tataricum* are the major source of flavonoid called rutin. Seeds of *F. tataricum* contains higher rutin content in comparison to *F. esculentum*. This review discusses the physiological and molecular basis of flavonoid biosynthesis and accumulation in plants in general and rutin and anthocyanin content in *Fagopyrum* species, its correlation with the expression of flavonoid pathway genes and the effect of different environmental factors on flavonoid biosynthesis. The understanding of rutin biosynthesis in buckwheat is expected to supplement for genetic improvement of buckwheat for higher nutritional value.

Keywords: biosynthetic pathway, comparative genomics, gene, rutin

Abbreviations: **4CL**, 4 Coumarate CoA ligase; **CHI**, Chalcone isomerase; **CHS**, Chalcone synthase; **C4H**, Cinnamate 4-Hydroxylase; **DAS**, day after seedling; **DW**, dry weight; **F3H**, Flavonol 3-Hydroxylase; **F3'H**, Flavonol 3'-Hydroxylase; **FLS**, Flavonone synthase; **PAL**, Phenylalanine ammonia lyase

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INTRODUCTION

Flavonoids constitute a relatively diverse family of aromatic molecules that are derived from Phenylalanine and malonyl-coenzyme A (CoA; via the fatty acid pathway). These compounds include six major subgroups that are found in most of the plants: the chalcones, flavones, flavonols, flavandiols, anthocyanins, and condensed tannins (or proanthocyanidins); a seventh group, the aurones, is widespread, but not ubiquitous (Winkel-Shirley 2001).

Buckwheat belongs to the family *Polygonaceae* and refers to two cultivated species: common buckwheat (*Fagopyrum esculentum*) and tartary buckwheat (*F. tataricum*). Buckwheat (*Fagopyrum* spp.), a pseudocereal, is a multipurpose food crop used both for grains and greens and has several medicinal and nutritional properties (Campbell 1997; la Casa *et al.* 2000; Landberg *et al.* 2011). Buckwheat contains several kinds of flavonoids in the seeds, leaves and stems, and rutin is one of these compounds (Couch *et al.*

1946). It is an antioxidant that has many useful pharmacological effects (Yildizoglu-Ari *et al.* 1991). Rutin is also present in substantial amounts in various other plants, such as apple, citrus, *Capris*, tomato, etc. (McGregor and McKilligan 1952; Attanassova and Bagdassarian 2009), and buckwheat is considered to be a major dietary source of rutin. Rutin is a flavonol with antioxidative and anti-inflammatory activities such as it has hypotensive effect, positive inotropic effect (Matsubara *et al.* 1985), strengthens the capillary blood vessels (Campbell 1997). Rutin can also be used as a natural coloring agent, an oxidation inhibitor, sunburn preventative in cosmetics (rutin absorbs ultra violet rays) and as an ingredient in functional food applications (WIPO 2004). The demand for rutin and other flavonoids derived from buckwheat is growing in the food, pharmaceutical and cosmetic industries due to its nutritional and medicinal value (Table 1).

The high level of rutin in buckwheat leaves and stems is accompanied by another member of the flavonoid family –

Table 1 Nutraceutical value of buckwheat.

Product/compound	Effect	Reference
Medicinal and nutritional value		
Gluten free proteins	Diet for celiac patients as an alternative to wheat	Drzewiecki <i>et al.</i> 2003
Rutin	Strengthens capillaries and helps in arteriosclerosis or high blood pressure	Campbell 1997
Rutin	Controls cholesterol	Kayashita <i>et al.</i> 1997
Rutin	Protection against gastric lesions	La Casa <i>et al.</i> 2000
Flavonoids	Anti-depressants, prevents signs of aging such as wrinkles and skin damage	Watanabe and Ayugase 2008
Buckwheat polyphenols	Ameliorate spatial memory impairment	Pu <i>et al.</i> 2004
Buckwheat protein	Suppresses gallstone formation and cholesterol level by enhancing bile acid synthesis	Tomotake <i>et al.</i> 2000
Phenolic antioxidants in buckwheat honey	Protects humans from oxidative stress	Schramm <i>et al.</i> 2003
Fagopyritol B1	Treatment of diabetes, polycystic ovary	Sattanathan <i>et al.</i> 2011
Buckwheat protein extract	Retard memory carcinogenesis by lowering serum estradiol, causes muscle hypertrophy	Kayashita <i>et al.</i> 1999
Buckwheat polyphenols	Treatment of polycystic ovary syndrome	Campbell 1997
Industrial value		
Flowers	Produces dark flavored honey having high antioxidant property	Saeger and Dyck 2001
Flour	Used in desserts, ice cream cones, dietetic foods, pancake mixes, canned meat products, canned vegetable products, and dried breakfast cereals	Bonafaccia <i>et al.</i> 2003
Flour	Making soba noodles as staple food in Japan	Taylor and Belton 2002
Grain	Baby food and in energy drinks	Fabjan <i>et al.</i> 2003
Tartary buckwheat raw material	Bitter buckwheat tea	Fabjan <i>et al.</i> 2003
Hull	Making pillows for relief of neck and back pain, muscle tension	Campbell 1997

anthocyanins, one of the final products of flavonoid biosynthetic pathways, which are water-soluble pigments in the leaves, stems, flowers, and roots. Anthocyanins play an important role in attracting insects or other animals for pollination and seed dispersal (Horbowicz *et al.* 2008). They also play a role as anti-oxidants and in protecting DNA and the photosynthetic apparatus from high radiation fluxes (Gould 2004). Anthocyanins have been associated with enhanced tolerance to chilling and freezing temperatures (Christie *et al.* 1994; McKown 1996; Hale *et al.* 2001; Nozzolillo *et al.* 2002; Leng and Qi 2003), to heavy metals (Krupa *et al.* 1996; Marrs and Walbot 1997; Hale *et al.* 2002) and to water stress (Sherwin and Farrant 1998; Farrant 2000; Farrant *et al.* 2003). The flavonoids, particularly proanthocyanidins in the seed coat, contribute to the maintenance of seed dormancy as well as increasing seed longevity in storage (Winkel-Shirley 1998; Debeaujon 2000; Debeaujon *et al.* 2003).

Flavonoids are also of significant interest as antioxidants and anticancer agents in the human diet (Rice-Evans 2001; Havsteen 2002; Stevens and Page 2004). Citrus flavonoids have many medicinal properties such as anticarcinogenic, anti-inflammatory and antioxidant (Stavric 1993; Elangovan *et al.* 1994) this also, resulted in reduction of coronary heart disease (Hertog *et al.* 1993; Di Majo *et al.* 2005). The interest in these classes of compounds is due to their pharmacological activity as radical scavengers (Cotelle *et al.* 1996). Park *et al.* (2000) demonstrated that buckwheat flowers have the potential of being used as a healthy food or as a medicine by detecting rutin in boiled water from the flowers. Clinical observations carried out on 75 diabetic patients treated with tartary buckwheat biscuits showed a decrease in the blood sugar level (Wang *et al.* 1992). Recent evidence suggests that certain flavonoids reduce dental caries and cariogenic bacteria incidence and are used as a promising natural agent for non-invasive root caries therapy (Wood 2007; Wu 2009). Flavonoids lower Alzheimer's disease (AD) by lowering amyloid β production, which plays an important role in AD (Paris *et al.* 2011). Flavonoids clearly have the potential to directly affect signaling and gene transcription through interaction with cytoplasmic and nuclear proteins. It has been suggested that flavonoids function in gene regulation in plants, for example, by inhibiting protein kinases that regulate the activity of transcription factors required for the synthesis of auxin transport proteins (DeLong *et al.* 2002; Buer and Muday 2004).

Buckwheat has been recognized as a healthy food because its seed is rich in vitamin B1 and B2, its protein has

high biological value (Sure 1955), proteins are particularly rich in lysine (6.1%), and contained less glutamic acid and proline and more arginine and aspartic acid than cereal proteins. Chemical analyses of buckwheat hydrolyzates indicated that the amino acid composition was nutritionally superior to that of cereal grains (Pomeranz and Robbins 1972)

SOURCES OF FLAVONOIDS

The flavonoids in different vegetables are as (mg/100 g of dry weight): broccoli 197, cauliflower 219, cabbage 147.5, red chili 829, lemon grass 178, black tea 1491, garlic 957, French bean 172.5, French peas 361 (Miean and Mohamed 2001). Of the *Allium* species, shallots and red onions represent the richest potential source of quercetin containing 95 and 64 mg/100 g, respectively. Quercetin 4'-glucoside and quercetin 3,4'-diglucoside are, in most cases, reported as the main flavonols in onions (*Allium cepa* L.) (Rune *et al.* 2007). The total flavonoid content of onion leaves (1497.5 mg/100 g quercetin, 391.0 mg/100 g luteolin, and 832.0 mg/100 g kaempferol) was followed by Semambu leaves (2041.0 mg/100 g), bird chili (1663.0 mg/100 g), black tea (1491.0 mg/100 g), papaya shoots (1264.0 mg/100 g), and guava (1128.5 mg/100 g) (Miean and Mohamed 2001). Green chilli pepper is one of the few vegetables that contain both flavonols (quercetin, 11.39 mg/100 g) and flavones (luteolin, 2.7 mg/100 g) at detectable levels. Celery and sweet ball peppers are the main food sources of flavones independent of flavonols (Janet and Garry 2006). Among the beverages, apple juice is one of the richest juice sources of catechins (containing 6.3 mg (-)-epicatechin/100 ml and 0.8 mg (b)-catechin/100 ml) whereas cranberry juice contains the most flavonols, mainly in the form of quercetin and myricetin (17.5 and 4.7 mg/100 ml, respectively) (Janet and Garry 2006).

Rutin has been identified in higher amount in *F. cymosum* (0.01%), *F. tataricum* (0.8-1.8%) and *F. esculentum* (0.01%) (Fabjan *et al.* 2003). On average, rutin content of *F. tataricum* sprouts was 2.2 fold higher than that of *F. esculentum* sprouts. Due to presence of flavonoids, high lysine content, crude fibre, vitamin B, buckwheat is considered as a nutraceutical food crop (Kim *et al.* 2006).

FLAVONOID BIOSYNTHESIS

Phenylpropanoids are a group of plant secondary metabolites derived from phenylalanine which are important for

many aspects of plant growth and development, such as pigment production, photoprotection, and disease resistance. The biosynthesis and accumulation of rutin and other flavonoids is controlled at the molecular level by structural and regulatory genes in different plant species (Winkel-Shirley 2001; Buer *et al.* 2010) and the pathway genes are conserved among different plant species and have been characterized at the genetic, biochemical and enzymatic levels in plant species including *Zea mays*, *Antirrhinum*, *Petunia*, *Arabidopsis* (Mol *et al.* 1998; Winkel-Shirley 2001). There have only been a few molecular studies of the flavonoid biosynthetic pathways in buckwheat. It is well known that rutin and anthocyanins share the same initial biosynthetic steps (Mol *et al.* 1998).

The flavonoid biosynthetic machinery has, until now, been found exclusively in the cytoplasm, where it appears to be organized as a multienzyme complex at the endoplasmic reticulum (ER). Fritsch and Grisebach (1975) suggested that flavonoid biosynthesis takes place partly or completely on membranes, based on analysis of enzyme activities in permeabilized cells and microsomes.

The first step in the pathway is catalyzed by phenylalanine ammonia lyase which is at the gateway from the primary metabolism into the important secondary phenylpropanoid metabolism in plants. Phenylalanine ammonia lyase (PAL) catalyzes the nonoxidative elimination of ammonia from L-phenylalanine (key precursors, obtained via shikimate and arogenate pathways, and malonyl-CoA derived from citrate produced by the TCA cycle) to give *trans*-cinnamic acid, a substrate common to the biosynthesis of different classes of phenylpropanoid products: anthocyanins, flavonoids, ultraviolet (UV) protectants, antimicrobial furanocoumarins, isoflavonoid phytoalexins, lignins and wound phenolic esters (Dixon and Paiva 1995; Ritter and Schulz 2004). Due to the central function of PAL at the branch point of phenylpropanoid derivative metabolism, this enzyme has been considered to be one of the key enzymes in the biosynthesis of flavonoids (Schijlen *et al.* 2004).

The second enzyme of the phenylpropanoid pathway, cinnamate 4-hydroxylase (C4H) is a member of the cytochrome P450 monooxygenase superfamily and it catalyzes the first oxygenation step during a phenylpropanoid metabolism, the hydroxylation of *trans*-cinnamate to *p*-coumarate (4-hydroxy *trans*-cinnamate). Next, 4-coumarate: CoA-ligase (4CL) converts *p*-coumarate to its coenzyme-A ester, which is a precursor for various phenylpropanoid biosynthetic derivatives, including lignins and flavonoids. Subsequently, chalcone synthase (CHS) catalyzes the production of a tetrahydrochalcone that is the precursor for all flavonoids. This step is the first dedicated reaction of the flavonoid biosynthesis pathway in higher plants. Lanz *et al.* (1991) have pointed out that this enzyme is well-conserved among plants of different groups, and that it has a cysteine residue at amino acid 169 that is thought to be part of the 4-coumaroyl-CoA binding site and which is required for enzyme activity. Afterward, chalcone isomerase (CHI) catalyzes the conversion of chalcone to naringenin, which is then converted to dihydrokaempferol and dihydroquercetin by flavone 3-hydroxylase (F3H) and flavonoid 3'-hydroxylase (F3'H), respectively. At this point, the pathway branches have two possible outcomes. In one branch, anthocyanidin synthase (ANS) catalyzes the conversion of leucoanthocyanidin to anthocyanidin (Holton and Cornish 1995; Pelletier and Shirley 1996; Shirley 1996; Wisman *et al.* 1998; Schoenbohm *et al.* 2000; Grotewold 2006). In the other branch, flavonol synthase (FLS) converts dihydroflavonols, such as dihydrokaempferol and dihydroquercetin, to flavonols, such as kaempferol and quercetin, respectively (Fritsch *et al.* 1981).

Rutin biosynthesis involves 9 genes, those of PAL, C4H, 4CL, CHS, CHI, FLS, F3H, Flavonol 3' hydroxylase (F3'H) and glucosyl/rhamnosyl transferase (3GT). Through a comparative genomics approach, genome information from related plant species was used to identify and characterize

the structural and regulatory genes involved in rutin biosynthesis in tartary buckwheat. (Chauhan *et al.* 2010). More than one fragment was isolated for C4H, F3H and F3'H, but only the fragments showing maximum identity with the genes from other plant species were selected for designing primers for gene expression analysis.

Primer pairs were designed from conserved regions of gene sequences retrieved from dicotyledon plants and amplified in *Fagopyrum* spp. (common, tartary and rice tartary buckwheat). Single band amplification was seen in CHS, 4CL and glucosyl/rhamnosyl transferases, whereas for F3H and C4H multiple copies of genes were amplified (Chauhan *et al.* 2010).

Isolation and sequence analysis of six anthocyanins biosynthetic genes was done in *F. tataricum*, which were cloned and characterized, namely, FtC4H, Ft4CL, FtCHI, FtF3H, FtF3'H, and FtANS, which encodes C4H, 4CL, CHI, F3H, F3'H, and ANS, respectively (Park *et al.* 2011).

SUBCELLULAR SITES / DISTRIBUTION OF FLAVONOIDS

Flavonoids are found in most plant cell compartments, including the cytosol, vacuole, ER, chloroplast, nucleus and small vesicles, as well as the extracellular space. Anthocyanins are cytotoxic and unstable in the neutral pH of the cytoplasm. Therefore, sequestration of anthocyanins into the acidic vacuole is an important component of the pathway leading to anthocyanin accumulation. Quercetin and kaempferol glycosides have been detected in the chloroplasts, which are capable of flavonoid biosynthesis (Hernández *et al.* 2009). In some tissues, such as the epidermis of leaves and flowers and endothelium of the developing seed coat, flavonoids are transported primarily to the vacuole by processes that appear to involve multidrug resistance-associated protein or multidrug and toxic compound extrusion proteins (Debeaujon *et al.* 2001; Mathews *et al.* 2003; Goodman *et al.* 2004).

Hrazdina and Wagner (1985) indicated that PAL, C4H, CHS, and UDP-glucose flavonoid glucosyltransferase (UFGT) function as part of one or more membrane-associated enzyme complexes in amaryllis, buckwheat, and red cabbage. Hrazdina (1992) stated that PAL, CHS, and UFGT were located in the cytosol, loosely associated with the cytoplasmic face of the endoplasmic reticulum (ER). An association of CHS with the cytoplasmic face of the rough ER (rER), but not with nuclei, plastids, mitochondria, Golgi, or tonoplasts, in buckwheat (*F. esculentum*) hypocotyls was also observed. In wild-type *A. thaliana* seedlings, flavonols accumulate in three main zones: the cotyledonary node, the hypocotyl-root transition zone and the root tip (Sheahan and Rehnitz 1993; Murphy *et al.* 2000; Peer *et al.* 2001; Saslowsky and Winkel-Shirley 2001). The wide distribution of flavonoids in plant cells and their probable biosynthetic sites on the cytosolic face of the ER imply that plants have efficient flavonoid transport systems with which to deliver these metabolites across various membrane-limited compartments. However, the transport mechanisms involved in trafficking of most primary and secondary metabolites are still poorly understood (Grotewold 2004). Two major hypotheses have been proposed for flavonoid transport: membrane vesicle-mediated transport and membrane transporter-mediated transport (Grotewold 2006). In vesicle-mediated flavonoid transport, anthocyanoplasts were first assumed to be transport vesicles or sites of anthocyanin biosynthesis (Markham *et al.* 2000; Grotewold 2004; Braidot *et al.* 2008). Anthocyanic vacuolar inclusions (AVIs) are more likely to be storage complexes than to be involved in anthocyanin transport. Membrane mediated transportation is carried out by ABC and MATE family transporters.

ESTIMATION OF RUTIN CONTENT IN DIFFERENT ORGANS AND GROWTH STAGES OF FAGOPYRUM SPECIES

Rutin content of tartary buckwheat is significantly higher than that of common buckwheat, with average values of 0.8 to 1.8% DW and 0.01% DW, respectively (Fabjan *et al.* 2003; Chauhan *et al.* 2010; Park *et al.* 2011). It is 300-fold more concentrated in groats (81 g/100 g) of tartary buckwheat than in those of common buckwheat (Steadman *et al.* 2001). In comparison, the amount of quercetin, another flavonoid present in buckwheat, is only 0.01-0.05% DW. Flowers are the richest source of rutin in buckwheat (6809 mg/100 mg DW) with 1000 times more rutin in comparison to roots (6.25 mg/100 mg DW) (Li *et al.* 2010). These results are consistent with previous studies that showed that the amount of rutin in buckwheat peaks at the full flowering stage (Dietrych-Szostak and Oleszek 1999; Gupta *et al.* 2011). There is more rutin in leaves positioned higher on the stem than in leaves at lower positions. Rutin concentration and rutin glucosidase activity by dry weight was high in young leaves (rutin content is more than 20% in un-expanded young leaves) and decreased along with the decrease of leaf position (Tatsuro *et al.* 2005). According to Kalinova and Dadakova (2006), the lowest amount of rutin was detected in buckwheat achenes and the plant parts, the richest in rutin were the leaves. However, amaranth inflorescences contained about half the amount of rutin compared to amaranth leaves (amaranth leaves contained up to 3% rutin per dry weight). According to Duke (1992), an amount of rutin similar to the level in amaranth (about 3%) is present in the leaves of parsley (*Petroselinum crispum*).

Amaranthus hybrid and *A. cruentus* were good sources of rutin. These species can provide 10–20 kg of rutin per hectare (in case of biomass production of about 2 t/ha DW) in a 60 days period of growth which is less than data obtained for common buckwheat. Kalinova and Dadakova (2006) determined rutin production of about 90 kg/ha (DW) at the flowering stage of buckwheat (about 60 days after sowing). In amaranth, about 45–90 kg/ha of rutin was obtained at the end of the growing period, which is due to the high production of biomass (about 30–45 t/ha), similar to the amount produced by common buckwheat.

Variation in rutin content in different plant parts of *Fagopyrum* spp. (*F. esculentum*, *F. tataricum*, *F. cymosum*) was reported as highest in the flowers and lowest in the roots (Park *et al.* 2004). Rutin content in inflorescences of tartary buckwheat was shown to be 9.5 times higher than in those of common buckwheat and 2.2 times higher than in those of *F. cymosum*. Rutin content in stems of tartary buckwheat was 34.9 times higher than in those of common buckwheat and 3.1 times higher than in stems of *F. cymosum*, while rutin in tartary buckwheat seeds was 3.2 times higher than in seeds of *F. cymosum*. Rutin content in plant parts of *F. tataricum* ranked in the order of flower > leaf > seed > stem > roots.

Differences in rutin content in seeds and in other tissues and growth stages of two *Fagopyrum* species, vis-à-vis expression profiling of flavonoid pathway genes has been reported by Gupta *et al.* (2011). Biosynthesis and accumulation of rutin showed significant variation among different growth stages from S1 to S9 (seed germination to seed maturation) of the *Fagopyrum* species, namely *F. tataricum* (IC-14889, IC-329457) and *F. esculentum* (IC-540858). Accessions of *F. tataricum* showed more or less similar pattern of rutin biosynthesis and accumulation in different growth stages to that of *F. esculentum*. Rutin content was higher during seedling stages of *F. tataricum* (3.6- to 4.6-fold) compared to *F. esculentum* and then increased exponentially from stages S3 to S6 (different leaf maturing and flowering stage) of *F. esculentum*, whereas the biosynthesis and accumulation of rutin showed a zigzag pattern during stages S2 to S6 of both the accessions (IC-14889, IC-329457) of *F. tataricum*. The dynamics of rutin biosynthesis and accumulation in different growth stages of both the

Fagopyrum species suggested that the higher amounts of rutin starts accumulating during post-flowering stages of *F. tataricum* as seed maturing stages of *F. tataricum* contains 40-50x higher rutin than *F. esculentum*.

ANTHOCYANINS IN BUCKWHEAT AND THEIR ACCUMULATION

The petals and sprouts of a common buckwheat cultivar (*F. esculentum*) contain four anthocyanins: cyanidin 3-*O*-glucoside, cyanidin 3-*O*-rutinoside, cyanidin 3-*O*-galactoside and cyanidin 3-*O*-galactopyranosyl-rhamnoside (Kim *et al.* 2007). Total amount of anthocyanins increases with flower development and the concentration of anthocyanins in the petals of buckwheat determine the colour of the flowers. For example, the 'Gan-Chao' cultivar which has red flowers, contains 4.69 µg of anthocyanins/petal, while the cultivar 'Kitawasesoba' which has white flowers, contains 0.06 µg of anthocyanins/petal.

All the organs of 'Hokkai T10' contained 2.6-6 times more anthocyanins than those of 'Hokkai T8' (Park *et al.* 2011). Hence the total anthocyanin content was higher in 'Hokkai T10' than in 'Hokkai T8', which is consistent with previous studies (Suzuki *et al.* 2009) which showed that anthocyanin accumulation was cultivar specific. These cultivar specific differences may be due to differential gene expression in different plant organs (Park *et al.* 2011); however, naringenin chalcone, a flavonoid, was absent from 'Hokkai T10' seedlings based on fluorescence microscopy, hence the accumulation of flavonoids and anthocyanins are inversely related in 'Hokkai T8' and 'Hokkai T10'. In terms of quantity, the major anthocyanin compound in buckwheat sprouts is cyanidin 3-*O*-rutinoside (Watanabe 2007). Cyanidin 3-*O*-glucoside and cyanidin 3-*O*-rutinoside concentrations in 6–10 day seedling sprouts of 'Hokkai T10' ranged from 0.16 to 0.20 mg/g DW and from 5.55 to 6.57 mg/g DW, respectively. In addition, dark-grown sprouts of 'Hokkai T10' accumulated 0.091 and 2.77 mg/g DW of cyanidin 3-*O*-glucoside and cyanidin 3-*O*-rutinoside whereas other varieties/breeding lines accumulated trace amounts of anthocyanins (Kim *et al.* 2007).

EXPRESSION ANALYSIS OF FLAVONOID (RUTIN) BIOSYNTHESIS GENES AT DIFFERENT GROWTH STAGES IN FAGOPYRUM SPECIES

The expression of flavonoid biosynthesis regulatory genes appears to be highly dependent on tissue type and/or response to internal or external signals which affect the signal transduction and gene expression involved in biosynthesis (Tsukaya *et al.* 1991; Dixon and Paiva 1995; Leyva *et al.* 1995; Mol *et al.* 1996; Laura *et al.* 2007; Ferri *et al.* 2009). The type and amount of flavonoids in plants depends on genotype and developmental stage (Hahlbrock and Grisebach 1979). Expression of flavonoid pathway genes was compared in *F. esculentum* (IC-540858) and *F. tataricum* (IC-329457) and their correlation with rutin content was reported by Gupta *et al.* (2011). Rutin content vis-à-vis expression analysis was carried out in different growth stages of *Fagopyrum* species. Out of 9 genes, expression levels of C4H, 4CL, GT, F3'H and F3H genes were not significant, whereas four genes, namely PAL, CHS, CHI and FLS showed differential expression with relatively higher amounts of transcripts in rice tartary buckwheat compared to common buckwheat during different growth stages. A positive correlation was observed between the expression of PAL, FLS, CHS and CHI genes and the rutin content, as there was a significant increase in these gene transcripts during the S6 (inflorescence) stage of *F. tataricum* compared to *F. esculentum*. PAL and CHS genes were highly expressed in the mature seeds of *F. tataricum* in S9, which results in higher rutin content than *F. esculentum* (*F. tataricum* contained 43-55x higher rutin compared to *F. esculentum*). CHI transcript level is higher in S7 (early seed development) in *F. tataricum* as compared to *F. esculentum*.

According to Park *et al.* (2011), the gene transcripts for all of the enzymes of the flavonoid biosynthetic pathway were expressed in every organ of *F. esculentum*, the expression levels were the highest in the stems and roots. The expression level of FePAL in the stems and roots was higher than in the flowers and leaves. In contrast to C4H, which was strongly expressed in all organs (flower, stem, leaf and root), FeF3H, FeF3'H, FeDFR, FeFLS1, FeFLS2, and FeANS were expressed at low levels in all these organs. In addition, FeFLS2 was expressed at very low levels in the roots, unlike FeFLS1.

EXPRESSION OF ANTHOCYANIN BIOSYNTHETIC GENES IN DIFFERENT ORGANS OF *F. TATARICUM*

Gene expression was compared in different organs and developmental stages of tartary buckwheat cultivars 'Hokkai T8' and 'Hokkai T10' (Park *et al.* 2011). Anthocyanin content was directly correlated with the expression of flavonoid biosynthesis genes. During flowering and seed ripening the FtANS gene was more highly expressed than other genes. Among the various genes for the anthocyanin pathway, the highly expressed genes were FtPAL, FtC4H, Ft4CL, FtCHS, FtCHI, FtF3H and FtANS in the flowers (for example the total anthocyanin content of the flowers of 'Hokkai T10' was 4.5 times that of 'Hokkai T8'), FtPAL, FtCHS and FtANS in the leaves, FtPAL, Ft4CL, FtC4H, FtCHS and FtANS in the stem, and FtC4H, Ft4CL, FtCHS, FtF3H and FtANS in the roots. During flowering and seed ripening the FtANS gene was more highly expressed than the other genes.

Quantitative real-time PCR analysis showed that these biosynthetic genes are more highly expressed in the lower parts of the plant (*i.e.* stems and roots) than in the higher parts of the plant (*i.e.* flower and leaves). There is an inverse relationship between the expression of flavonoid biosynthetic genes and the accumulation of their products in *F. tataricum*, similarly to *F. esculentum*. This may be due to transport of flavonoids within *Fagopyrum* species (Li *et al.* 2010; Park *et al.* 2011). Similar results of transport and accumulation were found in other plants. In *Arabidopsis* roots flavonoids were also found in the stele, which did not contain detectable levels of CHS or CHI, suggesting that flavonols may be transported between cells (Saslowky and Wink-Shirley 2001). The expression of AsPAL and AsC4H transcripts in *Allium sativum* was highest in the roots but surprisingly low in the bulbils, where phenylpropanoid compounds are most concentrated. These results suggest that some phenylpropanoids are synthesized in the roots and subsequently transported to the bulbils of *A. sativum* (Tuan *et al.* 2010).

ENVIRONMENTAL FACTORS AFFECTING FLAVONOID BIOSYNTHESIS, TRANSPORT AND ACCUMULATION

Cultivar and environmental factors, such as soil and climate, including temperature, UV radiation, sunlight, as well as cultural practices like sowing time and fertilizer also have an effect on the flavonoid content of plants. Individual biosynthetic genes may be regulated in response to a number of developmental and environmental signals. For example, in flowers the biosynthetic genes change their activities as a consequence of light and spatio-temporal developmental factors for the production of anthocyanins in the petal epidermal cells, coincidentally with flower fertility (Braidot *et al.* 2008). *Ginkgo biloba* GbPAL was also observed to be induced by a variety of stresses including UV-B, wounding, cold and salicylic acid. PAL is a key enzyme in plant stress response. Its biosynthesis is stimulated on pathogenic attack, tissue wounding, UV irradiation, low temperature, or low levels of nitrogen, phosphate, or iron (Dixon and Paiva 1995). The enzyme is accumulated in the vicinity of the affected tissue (Mauch-Mani and Slusarenko 1996; Ehness *et*

al. 1997).

A decrease of flavonoid biosynthesis has been observed when either endogenous (e.g. plant hormones), or exogenous factors (e.g. water and temperature stress, light, fertilizer, etc.) are limiting or excessive. In particular, plant hormones could affect flavonoid biosynthesis in a complex way. Abscisic acid, auxin and ethylene are responsible for an increase of flavonoids, while gibberellic acid and inhibitors of the ethylene receptor decrease their synthesis (Deikman and Hammer 1995; Dan and Lee 2004; Jeong *et al.* 2004). The gene expression is induced by sucrose, jasmonic acid and light irradiation, leading to an enhanced anthocyanin accumulation (Braidot *et al.* 2008). Methyl jasmonate inhibits anthocyanin biosynthesis and accumulation in hypocotyls of seedlings of common buckwheat (*F. esculentum* Moench) (Horbowicz 2008).

Light/UV light

Arabidopsis roots grown in complete darkness do not accumulate flavonoids since the expression of genes encoding enzymes of flavonoid biosynthesis are light-dependent (Charles *et al.* 2007). Different light exposures of fruits demonstrate that shading decreases significantly the flavonoid content of the berries. Long light hours could stimulate the increase of flavonoid content (Kim and Lee 2002), as flavonoid content of common buckwheat subjected to long sunlight hours was two times higher than that subjected to short sunlight hours. The total rutin content of buckwheat plant was highest under the natural light, intermediate under the blue-light, and lowest under red-light. The amount of rutin is also affected by the light and dark conditions (Suzuki *et al.* 1987; Campbell 1997), whereas other flavonoids, except gallic acid, are not significantly affected. In sprouts grown under light condition, rutin content (556.71 mg/100 g at 12 DAS) was 60% more than grown under dark conditions (343.25 mg/100 g at 12 DAS) (Park *et al.* 2011). The light-dependent biosynthesis would be limited only to flavonols, a result that is consistent with the role that these molecules play in protecting tissues from UV light (Downey *et al.* 2004). Expression of genes of flavonoid synthesis, except PAL and StSy, is coordinately enhanced by light in grapes (*Vitis vinifera*) (Sparvoli *et al.* 1994). Blue-light significantly decreased stem length, the number of tillers and number of nodes in both common and tartary buckwheat cultivars.

Flavonoid biosynthetic genes are induced by a Blue/UV-B Light Receptor. UV-B radiations alter the enzyme activity or gene expression and hence affect the flavonoid content of the plant. Several studies, mostly on CHS gene expression, have shown that the production of flavonoid and anthocyanin compounds in response to light is controlled, at least in part, at the level of transcription (Feinbaum and Ausubel 1988; Taylor and Briggs 1990; Feinbaum *et al.* 1991). For example, in parsley tissue culture cells CHS gene expression has been shown to be regulated by a UV-B light receptor, a blue light receptor, and phytochrome (Bruns *et al.* 1986; Ohl *et al.* 1989). UV-B was able to induce the activity of diphenylethyl phthalocyanine and the synthesis of rutin, and was also capable of inducing the accumulation of rutin in the organs (Kreft *et al.* 2002; Suzuki *et al.* 2005a). Tartary buckwheat leaf treated with stress using UV-B radiation, cold and desiccation showed an increase in rutin concentration of 122% by UV-B radiation and 129% by desiccation, whereas rutin glucosidase activity was increased 363% by UV-B radiation, 190% by cold treatment and 158% by desiccation treatment over the control. Hence, it was proposed that rutin and rutin glucosidase activity may be related to enhancement of the defense system against stress conditions in tartary buckwheat leaf (Suzuki *et al.* 2005a). When common buckwheat was exposed to reduced, ambient, and enhanced UV-B radiation, the total amounts of UV-absorbing compounds (rutin, quercetin, quercitrin and other flavonoids) were lower in buckwheat grown under reduced UV-B, as compared to

those under the ambient and enhanced radiation (Germ 2004). Modest amount of UV-B radiation may stimulate the synthesis of rutin, however, buckwheat plants may be damaged by higher doses (Gao *et al.* 2002; Krefth *et al.* 2002).

Temperature

The “accumulation of anthocyanin is more a function of temperature than of light”. Furthermore, anthocyanin content seems to be sensitive to diurnal differences in temperature, being higher in the presence of colder nights with respect to constant (high) temperature (Downey *et al.* 2006). More rutin is synthesized at higher average or day-night temperature (24.5°C daytime, and 18°C at night) in comparison to lower temperature (18°C daytime, and 12°C at night) (Schneider *et al.* 1996). Effect of temperature during the growth of strawberry on antioxidant capacity in the plant was studied by Wang and Zheng (2001), and it was found that high temperature conditions significantly enhanced the content of *p*-coumaroylglucose, dihydroflavonol, quercetin 3-glucoside, quercetin 3-glucuronide, kaempferol 3-glucoside, kaempferol 3-glucuronide, cyanidin 3-glucoside, pelargonidin 3-glucoside, pelargonidin 3-rutinoside, cyanidin 3-glucoside-succinate, and pelargonidin 3-glucoside-succinate in strawberry juice. Plants grown in the cool day and cool night temperature (18/12°C) generally had the lowest phenolic acid, flavonols, and anthocyanins. An increase in night temperature from 12 to 22°C, with the day temperature kept constant at 25°C, resulted in a significant increase in phenolic acid, flavonols, and anthocyanins. These conditions also resulted in a significant increase in the antioxidant capacity. The effects of temperature on rutin content of seedling, leaf and seed of buckwheat were studied by Chen and Wen (2005) in a growth chamber. The results showed that rutin content of the seedling was significantly higher than that of the seed, and it decreased with the increasing temperature. The rutin content of leaf and seed increased with the increasing temperature during the grain-filling stage.

As an essential factor for plant growth and metabolism, sugars are not only energy sources and structural components, but also are physiologic signals regulating the expression of a variety of genes involved both in primary and secondary metabolism (Koch 1996). Higher level of sugars – the first product of photosynthesis (and light) – could be the main reason for increased ANS in some plants exposed to light (Weiss *et al.* 1995; Vinterhalter *et al.* 2007; Horbowicz *et al.* 2008; Li *et al.* 2011). Boss *et al.* (1996) reported that the expression of 7 genes involved in anthocyanin biosynthesis was enhanced during the development of berry skins in *V. vinifera* under the influence of sugars.

Influence of sucrose on rutin content and flavonoid biosynthetic gene expression in seedlings of common buckwheat was studied by Li *et al.* (2011) The growth of buckwheat was inhibited when the concentration of sucrose was increased to 50 g/L in seedlings; however, the expression of most flavonoid biosynthetic genes was increased after 1 or 2 days of treatment and rutin content showed a marked increase when the concentration of sucrose was increased from 10 g/L to 50 g/L. Series of genes, namely, FeC4H, FeCHS, FeF3H, FeFLS1, FeFLS2 and FeANS was up-regulated during sucrose treatment (Li *et al.* 2011).

Fertilizers and water stress

Rutin content and the PAL activity were higher with increasing amounts of nitrogen application in grapevine. The highest values were detected at a nitrogen fertilizer level of 225 kg/ha. The rutin content and the PAL activity in the NPK combined application treatment were higher than in the NP, NK, PK. The changing trends in rutin contents were rather similar to PAL activity, which indicates that the rutin content was closely correlated with the PAL activity in buckwheat (China papers 2010). Flavonoid accumulation was induced by exposing plants for one week to nitrogen

depletion at 10°C, giving high levels of anthocyanins and the 3-glucoside-7-rhamnosides, the 3,7-di-rhamnosides, and the 3-rutinoside-7-rhamnosides of kaempferol and quercetin in buckwheat (Horbowicz *et al.* 2008).

Effects of water stress on flavonoids, rutin and quercetin content of seedlings of tartary buckwheat were studied by Na *et al.* (2008). The results showed that the quercetin content was very low in buckwheat seedlings. The rutin and flavonoid content of the seedling increased under water stress. Anthocyanin accumulation is also modified by the water status of the plant. During water stress, the synthesis of anthocyanins is paralleled by an increase of the expression of flavonoid transporter(s) (Braidot *et al.* 2008).

TRANSCRIPTIONAL REGULATION OF FLAVONOID BIOSYNTHESIS

Several transcription factors involved in the regulation of metabolic pathway genes have been isolated and studied. There are indications that transcription factor activity itself is regulated by internal or external signals leading to controlled responses. Regulators belonging to different transcription factor families, including WD40 (beta-transducin repeat), WRKY, basic-leucine zipper (bZIP), MADS-box, R2R3-MYB and basic helix-loop-helix (bHLH) factors, are involved in the transcriptional control of flavonoid biosynthesis genes (FBGs) and are reviewed in the work of Ramsay and Glover (2005).

The transcriptional control of flavonoid biosynthesis has been intensively studied (Broun 2005), and several classes of transcriptional regulators have been identified. The first group includes four MYB (myeloblastosis) proteins, production of anthocyanin pigments PAP1, PAP2, MYB113, and MYB114. Overexpression of any one of these, results in an increase of anthocyanin accumulation (Borevitz *et al.* 2000; Gonzalez *et al.* 2008). The second group encodes three redundant basic helix-loop-helix (bHLH) factors, transparent testa-8 (TT8), glabrous-3 (GL3), and enhancer of glabra-3 (EGL3), with their simultaneous inactivation causing anthocyanin deficiency (Zhang *et al.* 2003). MYB and bHLH proteins combine with the WD40 repeat-containing protein transparent testa glabra1 (TTG1) to form a transcriptional complex that activates anthocyanin biosynthetic genes, including anthocyanidin synthase (ANS), DFR, F3'H, leucoanthocyanin dioxygenase, UDP-glucosyl transferase 78d2 (UGT78D2), and UDP-glucosyl transferase 75c1 (UGT75C1) (Gonzalez *et al.* 2008). Three other closely related MYB proteins, MYB11, MYB12, and MYB111, regulate early steps of the flavonoid pathway, including those catalyzed by the enzymes encoded by CHS, CHI, F3H, and FLS1 (Stracke *et al.* 2007). In addition, MYBL2, an R3-MYB-related protein, acts as a repressor by interfering with the formation of the MYB-bHLH-WD40 complex (Dubos *et al.* 2008; Matsui *et al.* 2008). It has been reported that the transcription factor genes MYB75/PAP1 and PAP2/MYB90 play an essential role in the sucrose-induced anthocyanin biosynthesis pathway (Lloyd and Zakhleniuk 2004; Teng *et al.* 2005). It is possible that such regulatory genes have a close relationship and contribute to the increase in rutin synthesis (Li *et al.* 2011).

The fine regulation of flavonoid biosynthesis is achieved by combinatorial action(s) of transcription factors, expressed in a spatially and temporally controlled. MYB factor PAP1, but not PAP2, strongly stimulates the expression of the anthocyanin structural gene encoding dihydroflavonol reductase, but neither factor affected the expression of the early flavonoid biosynthesis gene encoding chalcone synthase. All bHLH genes (TT8, EGL3 and GL3) showed light induction, and in seedlings their expression preceded that of the late structural genes, suggesting their possible role in light regulation of these structural genes. The first functional characterization of a light-inducible MYB transcription factor controlling flavonol synthesis was studied in developing grape berries. Flavonol-spe-

cific MYB transcription factor VvMYBF1 activates promoters of the flavonoid pathway genes VvCHI, VvCHS and VvFLS1 required for flavonol synthesis in fruits (grapevine) (Stefan *et al.* 2009). *Arabidopsis* HY5 encodes a bZIP factor that is a key positive regulator of light signaling during plant development, and that regulates numerous genes during photomorphogenesis, including AtCHS, AtFLS and AtMYB12 (Lee *et al.* 2007). ANAC078 protein is associated with the induction of genes related to flavonoid biosynthesis (the transcript levels of PAP, AtMYB4, CHI, F3H and EGL3 was higher), leading to the accumulation of anthocyanins, in response to HL stress. In *Arabidopsis*, members of the AP2/ERF (apetala2/ethylene response factor), bZIP, NAC, (homeodomain and leucine zipper) HD-ZIP, and MYB/MYC families, as well as several classes of zinc finger domain proteins, are induced by drought stress (Shinozaki *et al.* 2003; Zhang *et al.* 2004). The *Arabidopsis* NFYA5 (Nuclear transcription factor Y subunit A-5) transcription factor is regulated transcriptionally and posttranscriptionally to promote drought resistance (Wen-Xue *et al.* 2008), and related transcription factor, NFYB1, was reported to confer drought tolerance not only in *Arabidopsis* but also in maize, when overexpressed. The utility of NFYB1 (Nuclear transcription factor-Y beta) overexpression is in stabilizing crop yield under drought conditions (Nelson *et al.* 2007).

CONCLUSION

The biosynthesis and accumulation of flavonoids in different growth stages of *Fagopyrum* species along with the expression of flavonoid biosynthetic genes, would help in understanding the physiological and molecular dissection of high rutin content in *F. tataricum*. The transcription factors potentially involved in rutin accumulation and seed maturation in *Fagopyrum* species still not known. No information on genes contributing in biosynthesis, modification, transport and accumulation of high rutin content in tartary buckwheat is there. Identification of candidate genes for various traits of economic importance, including those involved in the biosynthesis of rutin and other secondary metabolites would be useful for enhancing medicinal and nutritional properties of buckwheat both by conventional and non-conventional plant breeding methods

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