Molecular Dissection of Flavonoid Biosynthesis Machinery in a Nutraceutical Food Crop (*Fagopyrum* spp.)

By

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CERTIFICATE

This is to certify that the thesis entitled **"Molecular Dissection of Flavonoid Biosynthesis Machinery in a Nutraceutical Food Crop (*Fagopyrum* spp.)"** submitted by **Mrs Nidhi Gupta** to the Jaypee University of Information Technology, Waknaghat in fulfillment of the requirement for the award of the degree of **Doctor of Philosophy in Biotechnology & Bioinformatics** is a record of bona fide research work carried out by her under my guidance and supervision and no part of this work has been submitted for any other degree or diploma.

(Dr. R.S. Chauhan)

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(Nidhi Gupta)

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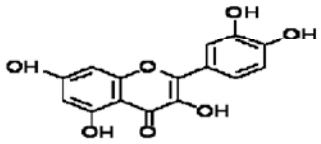
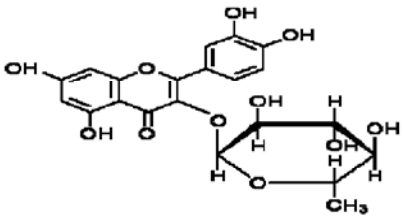
Fagopyrum esculentum



Quercetin



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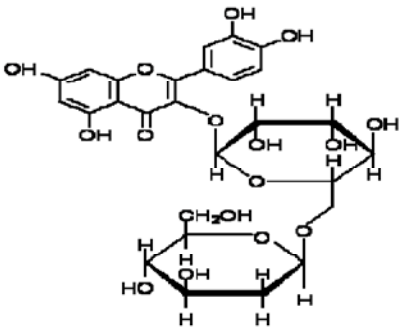

Rutin

Major Secondary metabolites (flavonoids) present in *Fagopyrum* species

Nutraceuticals have off late received considerable interest because of their potential nutritional and therapeutic effects. Nutraceuticals (foods with nutritional and medicinal value), are being projected as functional foods due to desirable therapeutic outcomes with reduced side effects as compared to other therapeutic agents. Some popular nutraceuticals include buckwheat, oat, barley, flaxseed, psyllium, brown rice, soy and products, omega-3 eggs, calcium-enriched orange juice, green tea, etc. (Saikia and Deka 2011). The use of nutraceuticals, as an attempt to accomplish desirable therapeutic outcomes with reduced side effects, as compared with other therapeutic agents has met with great success (Rajasekaran et al., 2008). The major active nutraceutical ingredients in plants are flavonoids which represent a large family of low molecular weight polyphenolic secondary metabolites that are widespread throughout the plant kingdom, ranging from mosses to angiosperms (Winkel-Shirley 2001; Tapas et al., 2008). Over 6,000 flavonoids have been identified, and categorized, according to chemical structure, into flavonols, flavones, flavanones, isoflavones, catechins, anthocyanidins, dihydroflavonol and chalcones (Lehne and Saliba 2008; Sandhar et al., 2011). Flavonoids promote physiological survival of plant by protecting it from pathogenic micro organisms and UV radiations. In addition, flavonoids are involved in pigmentation for flowers, fruits and seeds to attract pollinators and seed dispersers, morphogenesis, sex-determination and in plant-microbe interactions (Winkel-Shirley 2001; Grotewold 2006; Sandhar et al., 2011; Zhou et al., 2011a). Flavonoids also contribute to nutraceutical qualities of fruits and vegetables and have long been recognized to possess anti-oxidant, anti-inflammatory, anti-allergic, hepato-protective, anti-thrombotic, antiviral, and anti-carcinogenic activities (Grotewold 2006; Tapas et al., 2008). The health-promoting effects of flavonoids may relate to interactions with key enzymes, signaling cascades involving cytokines and transcription factors, or antioxidant systems (Buer et al., 2010).

One of the bioactive flavonoid, rutin, is present in substantial amounts in various plant species such as *Viola tricolor* (3.36%), *Cappris spinosa* (0.28%), Apple (0.17%), *Lycopersicon esculentum* (0.002%-0.009%) and many more (McGregor and McKillican 1952; Attanassova and Bagdassarian 2009; Sofic et al., 2010). The use of rutin as a medicinal agent for the treatment of vascular disorders characterized by abnormally fragile or permeable capillaries has stimulated interest in this compound (Campbell 1997). In addition to its ability to reduce hemorrhage in people with high blood pressure, rutin has several other medicinal properties, including pharmacological, vasconstrictive, spasmolitic and positive inotropic effects (Campbell 1997; La Casa et al., 2000; Schramm et al., 2003; Wang et al., 2009a). Therefore, due to various medicinal and nutritional properties of rutin and other flavonoids, their demand is growing in the food, pharmaceutical and cosmetic industries (Wang et al., 2009a; Kunti´c et al., 2011; Sandhar et al., 2011).

Buckwheat (*Fagopyrum* spp.), is a pseudo-cereal having many medicinal and nutraceutical properties, is the only food crop known as the richest source of rutin (McGregor and McKillican 1952). Buckwheat is a multipurpose crop used for both grains and greens (Campbell 1997). The genus *Fagopyrum* belongs to the family *Polygonaceae* and has 20 known species which mainly occur in the highlands of Euro-Asia (Arora and Engels 1992; Ohnishi 1995; Shao et al., 2011). Of these, two cultivated species, *F. esculentum* (Common buckwheat) and *F. tataricum* (Tartary buckwheat) are of high economic importance due to multiple uses such as a substitute for cereals in human consumption, as a vegetable crop, honey crop, and of ethno-botanical importance (Li and Zhang 2001; Schramm et al., 2003). Owing to the high lysine content, buckwheat proteins have a higher biological value than the cereal proteins, such as those of wheat, barley, rye and corn. The main protein solubility fraction in buckwheat is globulin

(Eggum et al., 1981). It is gluten free, thus making it a valuable nutrient in the diets of people who are sensitive to gluten (Drzewiecki et al., 2003; Sedej et al., 2011).

The difference in morphology of seeds of *F. esculentum* and *F. tataricum* reveal that the fruits of common buckwheat are triangular, large 3 edged achene in comparison to tartary buckwheat where fruits are small, ovoid, and conical with dull irregular faces on each side (Figure 1.1; Léder 2002). The nutritional composition of buckwheat seeds has been well studied (Bonafaccia et al., 2003). Buckwheat accounts for 70% of total carbohydrates with starch as the major component which is 73% in common buckwheat seeds and 70% in tartary buckwheat seeds (Campbell 1997). In tartary buckwheat, fagopyritols (mono-, di- and trigalactosyl derivatives of D-chiro-inositol) account for 40% of total soluble carbohydrates compared to 21% in common buckwheat thus, helps in the treatment of diabetes (Steadman et al., 2000). Total flavonoids are relatively higher in tartary buckwheat (40mg/g) compared to common buckwheat (10mg/g) of which rutin is the major component (Li and Zhang 2001). Tartary buckwheat seeds contain more rutin (about 0.8 to 1.7% DW) compared to common buckwheat seeds (0.01% DW) (Fabjan et al., 2003). Total B vitamin content is 2 times higher in tartary buckwheat than in common buckwheat (Bonafaccia et al., 2003). Hence, tartary buckwheat is considered an excellent food material with a potential for preventive nutrition due to the presence of proteins with high biological value (90%) and balanced amino acid composition, relatively higher crude fiber, and Vitamin B compared to common buckwheat. But, the seeds of tartary buckwheat have a tightly adhering hull that makes it difficult to de-hull and contains a bitter component that affects its palatability (Campbell 1997). Rice-tartary buckwheat is a type of tartary buckwheat with a non-adhering hull property that can be a potential nutraceutical food source (Figure 1.1; Wang and Campbell 2007). It has also been reported that this type of tartary buckwheat was

cultivated and used as a rice replacement in the staple diet in Nepal, Bhutan and southern China (Campbell 2003).

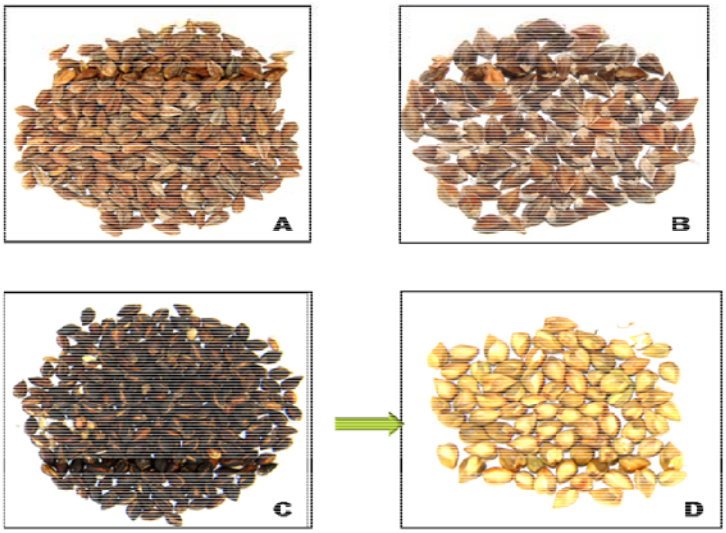

Figure 1.1 Seed morphology of tartary (A), common (B) and rice-tartary buckwheat (C). Non-adhering hull of rice tartary buckwheat removed (D).

Contrary to most of the medicinal and aromatic plants prevalent in the Himalayas, the *F. tataricum* has an added advantage for investigating the molecular genetics of secondary metabolite production due to its annual growth habit with short life cycle, diploid nucleus, self fertility and higher seed set (Chauhan et al., 2010). Variation for rutin content or other flavonoids is highly desirable not only for identifying high content accessions but also to explore for novel genes/ alleles contributing to higher flavonoids content. Variation for rutin content has been studied on a limited scale among *F. tataricum* genotypes. Yan et al. (2004) showed significant variation in rutin content among 14 genotypes of tartary buckwheat (0.87-1.4% rutin) and common buckwheat (0.4-0.5% rutin) grown in different ecological conditions. Rutin content variation was also observed among 50 strains of tartary buckwheat on the basis of differences in

seed shape and color (Park et al., 2004). However, in those studies rutin content was characterized by testing mature seeds of different buckwheat genotypes collected from diverse ecological regions, which can be largely influenced by the environmental conditions (altitude, light intensity, temperature, etc.) rather than only the genotype of seed samples. Hence, we report rutin content variation among *F. tataricum* accessions need to be studied by growing at the same environmental conditions so as to reflect genotypic variation.

Various molecular markers (AFLP, RAPD, RFLP and SSRs) have been successfully used in the diversity analysis and utilization of plant genetic resources (Botstein et al., 1980; William et al., 1990; Vos et al., 1995; Zietkiewicz et al., 1994). The genetic relationship was studied among natural populations and cultivated landraces of tartary buckwheat through RAPD and AFLP markers (Kump and Jovornik 2002; Sharma and Jana 2002b; Senthilkumaran et al., 2007). Microsatellite markers were also used for diversity analysis in tartary buckwheat (Li et al., 2007). However, no efforts have been made till date to correlate genetic relationship with the high vs. low rutin content in accessions of buckwheat which can provide helpful information for evaluation, breeding and conservation of germplasm. Therefore, characterization of tartary buckwheat for rutin content variation vis-à-vis DNA fingerprinting was undertaken among a collection of *F. tataricum* accessions from the Himalayan regions of India.

The flavonoid content in a particular tissue and developmental stage is largely influenced by different classes of regulatory genes, transporters, modifiers etc in addition to structural genes of the flavonoid biosynthetic pathway (Grotewold 2006; Bovy et al., 2007; Hichiri et al., 2011). Understanding the molecular biology of high rutin content (40-50x) and nutritional superiority in seeds of tartary buckwheat compared to common buckwheat would provide information on what genes/alleles in *F. tataricum* are contributing to high rutin content help in planning. The lack of

basic genome resources such as genetic map, seed ESTs, genome libraries, molecular markers, etc. further complicates molecular dissection of flavonoid machinery in buckwheat. The biosynthesis and accumulation of flavonoids is controlled by the structural and regulatory genes, which are studied in different plant species such as *Zea mays*, snapdragon, *Petunia*, *Arabidopsis* (Winkel-Shirley 2001; Grotewold 2006; Hichiri et al., 2011). The cloning and differential expression of anthocyanin biosynthesis pathway genes in different tissues (leaves, stem and flowers) of *Fagopyrum* species was studied without looking at rutin content variation vis-à-vis genes controlling or regulating rutin biosynthesis (Li et al., 2010; Park et al., 2011a). Therefore, physiological and molecular dissection of higher rutin content in *F.tataricum* compared to *F.esculentum* and its correlation with the expression of flavonoid pathway genes or uncovering novel genes would provide avenues for genetic improvement of buckwheat for enhanced flavonoid (rutin) content.

The lack of whole genome sequence and non-availability of ESTs from developing seeds of *Fagopyrum* spp. prompted us to utilize differential transcript profiling through cDNA-AFLP to decipher what genetic factors, in addition to flavonoid structural genes, contribute to high rutin content of rice-tartary compared to common buckwheat. The cDNA-AFLP has been suggested as an efficient method for isolation and identification of differentially expressed genes (Bachem et al., 1996). The cDNA-AFLP has an added advantage of not only capturing differences in the abundance and types of transcripts but can also detect differences in the DNA sequences of transcript fragments in two *Fagopyrum* species.

Keeping in view the economic importance of buckwheat, lack of information on rutin content variation in genotypes/landraces prevalent in North-Western Himalayas of India, virtually no information on molecular basis of high rutin biosynthesis in tartary buckwheat, the current study was undertaken with the following objectives:

OBJECTIVES

1. Rutin content profiling and AFLP fingerprinting of *F. tataricum* accessions from North-Western Himalayas of India

2. Cloning flavonoid pathway genes through comparative genomics and their expression vis-à-vis rutin content variation in different growth stages of *F. tataricum*

3. Differential transcript profiling to capture genes regulating biosynthesis, modification, transport and accumulation of flavonoids in *Fagopyrum* spp.

Chapter-II

REVIEW OF LITERATURE

The current status of literature on various aspects of buckwheat research has been reviewed as under:

2.1) Buckwheat as a nutraceutical food crop

Buckwheat (*Fagopyrum* spp.) is a multipurpose food crop used for both grains and greens, known to have several medicinal and nutritional properties. Starch is the major carbohydrate in buckwheat, comprising 51% to 70% of the seed (Campbell 1997). Owing to the high lysine content, buckwheat proteins have a higher biological value than the cereal proteins of wheat, barley, rye and corn. The main protein solubility fraction in buckwheat is globulin. It is gluten free, thus making it a valuable nutrient in the diets of people who are sensitive to gluten. Buckwheat grains are an important source of microelements, such as: Zn, Cu, Mn, Se (Stibilj et al., 2004), and macroelements: K, Na, Ca, Mg (Wei et al., 2003). With 80% unsaturated fatty acids more than 40% are constituted by polyunsaturated fatty acid (PUFA) (Krkoškova and Mrazova 2005). The significant contents of rutin, catechins and other polyphenols as well as their potential antioxidant activity are also of significance to the dietary value (Oomah and Mazza 1996; Wanatabe 1998). Moreover, buckwheat grains are a rich source of TDF (total dietary fibre), soluble dietary fibre (SDF), and are used in the prevention of obesity and diabetes (Brennan 2005). Buckwheat seeds also accumulate fagopyritol B1, a galactosyl derivative of *D-chiro*inositol, in embryo and aleurone tissues. Fagopyritol B1 is the most prevalent of five fagopyritols identified in buckwheat seeds and is correlated to seed desiccation tolerance and potentially used as a dietary treatment for type II diabetes (Obendorf 1998). Rutin is the major

flavonoid present in buckwheat, has several medicinal properties including pharmacological, anti-arteriosclerotic, vasconstrictive, spasmolitic and positive inotropic effects (Campbell 1997; La Casa et al., 2000; Schramm et al., 2003; Wang et al., 2009a). Therefore, due to various medicinal and nutritional properties the demand of buckwheat is growing in the food, pharmaceutical and cosmetic industries (Kunti´c et al., 2011; Sandhar et al., 2011). Nutraceutical values of buckwheat are listed in Table 2.1.

Table 2.1 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 et al. (2003)
Rutin	Strengthens capillaries, helps in arteriosclerosis or high blood pressure	Campbell (1997)
Rutin	Controls Cholesterol	Kayashita et al. (1997)
Rutin	Protection against gastric lesions	La Casa et al. (2000)
Flavonoids	Anti-depresents, prevents signs of aging such as wrinkles and skin damage	Watanabe and Ayugase (2008)
Buckwheat polyphenols	Ameliorate spatial memory impairment	Pu et al. (2004)
Buckwheat protein	Suppresses gallstone formation and cholesterol leve by enhancing bile acid synthesis	Tomotake et al. (2000)
Phenolic antioxidants in buckwheat honey	Protects humans from oxidative stress	Schramm et al. (2003)
Fagopyritol B1	Treatment of diabetes, Polycystic ovary	Sattanathan et al. (2011)
Buckwheat protein extract	Retard memory carcinogenesis by lowering serum estradiol, causes muscle hypertrophy	Kayashita et al. (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 et al. (2003)
Flour	Making soba noodles as staple food in Japan	Taylor and Belton (2002)
Grain	Baby food and in energy drinks	Fabjan et al. (2003)
Tartary buckwheat raw material	Bitter buckwheat Tea	Fabjan et al. (2003)
Hull	Making pillows for relief of neck and back pain, muscle tension	Campbell (1997)

2.2) Genetic resources in buckwheat

The genus *Fagopyrum* belongs to the family *Polygonaceae* and has 20 known species which mainly occur in the highlands of Euro-Asia (Arora and Engels 1992; Ohnishi 1995; Shao et al., 2011). Of these, cultivated species *F. esculentum* and *F. tataricum* have wider distribution, while other species *F. homotropicum*, *F. caudatum*, *F. sagittatum*, *F. cymosum*, *F. megaearpum*, *F. gracilipes*, *F. urophyllum*, *F. leptopodum*, *F. lineare*, *F. callianthum*, *F. pleioramosum*, *F. capillatum, F. pugense, F. wenchuanense, F. dibotrys, F. qiangcai* and *F. statice* occur mainly in the highlands of Euro-Asia (Farooq and Tahir 1987; Anon 1988; Baniya 1995; Ohnishi 1995; Rana 2004). Furthermore, *F. tataricum* ssp. *annum* occurs in the Eastern Himalayas, *F. tataricum* ssp. *potanini* in Tibet, Kashmir Himalayas and northern Pakistan (Ohnishi 1989, 1991, 1992), while *F. tataricum* ssp. *himalianum* and *F. tataricum* ssp. *emarginatum* are distributed in the cold arid regions of Western Himalayas of India (Rana 2004). Munshi (1982) described *F. kashmirianum* as a separate taxon but morphologically akin to *F. tataricum*, hence treated as the same species. Important traits of *Fagopyrum* species are listed in Table 2.2.

Table 2.2 Characteristics of *Fagopyrum* species

Species	Features	Reference
F.homotropicum	Homostylous self compatible	Ohnishi and Matsuoka (1996)
F.tataricum	Homostylous self compatible, small genome size, frost tolerant, high rutin, quercetin, quercitrin, Fagopyritols	Fabjan et al. (2003); Campbell (1997)
F.urophyllum, F.statice	Perennial shrub	Ohnishi (1998)
F.cymosum	Perennial shrub, autotetraploidy, rutin	Ohnishi and Matsuoka (1996)
F.esculentum	Heterostylous self-incompatible, fagopyritols, rutin	Campbell (1997)
F.gracilipes	Homostylous self compatible, autotetraploidy	Kole (2011)
F. callianthum	Heterostylous, self fertilizing	Campbell (1997)
F. dibotrys	Perennial, rhizome is a traditional folk medicine, High protein content than other species	Lan et al. (2011)
F. qiangcai	Resembles like *F.esculentum*	Shao et al. (2011)

The current status of buckwheat genetic diversity collected and maintained in east and south Asia has been documented by the International Plant Genetic Resources Institute (now known as Biodiversity International) Regional Office for Asia, the Pacific and Oceania (IPGRl-APO 1999). Approximately 5,000 accessions of buckwheat species have been collected in east and south Asia, which consist of about 52% of the world's buckwheat collections. Nearly 90% of the world's tartary buckwheat accessions are native to Asia. China has the largest collection of buckwheat accessions (2146) followed by India (954), Japan (746), DPR Korea (413), Nepal (327), North Korea (95) and Mongolia (30) (IPGRl-APO 1999; Zhou and Zhang 1995; Zhang et al., 2004). Accessions of nearly 50 wild species are maintained by China, Japan and India (Zhang et al., 2004).

Buckwheat exhibits much variation in the distribution and diversity including those of wild species throughout the Indian Himalayan region with more preponderance in the western Himalayan region than the north-eastern region (Onishi 1995; Rana 2004). The occurrence of buckwheat ranged from Jammu and Kashmir in the north to Arunachal Pradesh in the east and Tamil Nadu in the South. The important areas where cultivation of buckwheat is more predominant are Kargil and Drass sectors, Gurez valley of Jammu and Kashmir; Bharmaur, Pangi, outer Saraj, Chopal, Dodra kuar, Neshang, Pooh subdivision, Lahaul valley, Pin valley in Himachal Pradesh; Pindari valley, Darma valley, Jolwan, Jaunpur, Kapkote in Uttranchal; Cooch Behar, Darjeeling in West Bengal; Lachan, Lachoong in Sikkim; Tawang, Bomdilla and Dirang in Arunachal Pradesh, other higher elevations of Meghalaya and Manipur and also sporadically in the Nilgiris and Palani hills in the southern part of India. The germplasm of buckwheat has been augmented both through collections made within the country and introduced from abroad. The present collection comprises 911 germplasm accessions consisting of *F. esculentum* (333),

F. tataricum (540) and other wild species (38), and is stored in the Medium Term Storage at 7°C temperature and 35% RH at National Bureau of Plant Genetic Resource (NBPGR), Regional Research Station, Shimla. In addition, 837 accessions have been conserved for long term storage at -20°C as base collection in the National Gene Bank at NBPGR, New Delhi. Apart from the NBPGR, about 300 working collections are also maintained at ambient temperature by other institutes such as the G. B. Pant Agricultural University Hill Campus, Ranichauri, Vivekananda Parvatiya Krishi Anusandhan Shala, Almora, and the Regional Stations of Agricultural University, Palampur at Sangla and Kukumseri.

Buckwheat germplasm has been evaluated for quality characters such as amino acids, vitamins E and P contents (Keli and Dabiao 1992; Suzuki et al., 2005; Lin et al., 2008). The variation for protein content (8.20 to 15.10%), total phenols (1.4-1.70%), free phenols (0.27-0.94%) and available lysine (3.89-5.60%) was reported in 60 accessions under All India Coordinated Research Project on Under-Utilized Crops (Anon 2008). Multi-location testing of 13 elite varieties showed that 'IC13374', 'IC13411', 'Kullu Gangri', and 'VL7' were the high yielding and most consistent varieties (Joshi and Rana 1995). Some of the promising accessions identified in the germplasm evaluated in India are given in Table 2.3.

Table 2.3 Promising germplasm identified for various yield contributing characters (Rana et al., 2011)

Characters	Germplasm accessions	Value
Leaf length	IC341081, IC274444, IC310104, IC278957, C274439	>10 cm
Leaf width	IC341681, IC311004, IC310046, IC318859, C310047	>10 cm
No. of leaves/plant	IC18889, EC125357, EC58322, IC329568, IC341656	>100
No. of internodes	IC258244, IC258230, IC341680, IC313468,IC381077	>20
No. of primary branches	EC188664, IC274423, IC318859,IC329194,IC318859	>8
Cyme length	IC361635, IC341631, IC318859, EC125357,EC58322	>7 cm
Days to maturity	IC310104, EC323731, IC341671, EC323729,IC329568	<80days
Seed yield/plant	IC18869, IC18889, IC318859, IC329401, IC329404	>100 g
1000 seed weight	IC381077, IC381098, IC381049, EC216685, IC58322	>25 g
Protein content	IC108499, IC108500, IC107989, IC291963,IC382287	>14.0%
Total phenols	IC.310045,IC274439, IC341674, IC266947,IC274439	>1.60%
Lysine	IC341674, IC310045, IC274439, IC108499,IC274438	>4.5%

2.3) Current status of genome resources in buckwheat

The development and use of molecular markers for the detection and exploitation of DNA polymorphism is one of the most significant developments in the field of molecular genetics (Cullis 2002). A genetic marker can be defined in one of the following ways: (a) a chromosomal landmark or allele that allows for tracing a specific region of DNA; (b) specific piece of DNA with a known position on the genome or (c) a gene whose phenotypic expression is easily distinguished, used to identify chromosomes, or locus. Genetic maps are used for detailed analysis of association between genes or quantitative trait loci (QTLs) and economically important traits and, thereby, aiding introgression of desirable genes or QTLs through marker-assisted selection (MAS). For this purpose, PCR-based methodology has been found more convenient than restriction fragment length polymorphism (RFLP) analysis because of the relative ease of detection and smaller amount of DNA required. Many fingerprinting techniques

based on PCR, such as SSRs or microsatellites, random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) has been developed over the past several years (Chauhan et al., 2010). Sequence characterized amplified regions (SCARs) and sequence tagged sites (STSs) are also types of PCR markers, but they differ from the former methods by having single or two bands in agarose gels.

Much of the progress on research in molecular genetics and plant breeding has been achieved using common buckwheat, and only fragmentary research efforts have been made in tartary buckwheat. Aii et al. (1998) developed SCAR markers that were closely associated with the *Sh* gene (homomorphic self-compatible having the middle-styled morph), and these proved useful in identification of heterozygosity (Aii et al., 1999). Using AFLP markers, Nagano et al. (2001a) exploited the F2 progeny of *F. esculentum* and *F. homotropicum* for fine mapping of the *Sh* allele (homostylar locus). Five AFLP markers linked to the *sht1* locus (genes linked to brittle pedicel in buckwheat) were identified (Matsui et al., 2004) and two of them were converted into STS markers, which were useful for MAS of non-brittle pedicel plants. An inter-specific linkage map using *F.esculentum* and *F. homotropicum* was developed (Yasui et al., 2004). The *F. esculentum* map had 8 linkage groups with 223 markers covering a total of 508.3 centi Morgan (cM), whereas the *F. homotropicum* map consisted of 211 markers covering 548.9 cM. Morphological markers, distylous self-incompatibility and shattering habit were tightly linked to each other (1.3 cM) and located near the center of linkage group 1. Another marker, winged seed, was located on linkage group 4. More refined genetic map of F2 progeny (n = 225) of hybrids between the Sobano variety of *F.esculentum* and the Homo wild accession (*F. esculentum* var. *F. homotropicum*) was developed using RAPD, STS and seed protein subunit markers, and three morphological traits (Pan and Chen 2010). Ten linkage groups were identified

in *F. esculentum*, involving 87 RAPD markers, 12 STS markers, four seed protein subunit (PS62/PS59, PS49.8/PS51.4, PS44/PS42.9, and PS39.9/PS37.8) markers, and three morphological alleles controlling homo/long style (*H/s*), shattering habit (*Sht/sht*), and acute/obtuse achene ridge (*Ac/ac*), covering a total of 655.2 cM (Pan and Chen 2010).

In common buckwheat, only 5 microsatellite markers have been developed by sequencing 2785 clones from the libraries. 1483 clones contained microsatellites, which were enriched for (CT)n and (GT)n repeats. Primer pairs were designed for 237 of the microsatellite loci, of which 54 primer pairs were highly variable. These primers were evaluated for their ability to detect variations in common buckwheat populations and utilized in 7 related *Fagopyrum* species, including *F.tataricum* (Konishi et al., 2006). Efforts have also been made to develop microsatellite markers in tartary buckwheat (Li et al., 2007), which included the construction of a genomic library enriched with (gT)n repeats by using 5′-anchored PCR for the development of microsatellite markers. Ma et al. (2009) reported 136 new SSR markers in *F.esculentum* and showed their application for diversity analysis in related species of the genus *Fagopyrum*. However, of these 136 SSRs, only 10 were polymorphic on 41 accessions of diverse species origin. The applicability of 17 EST primers developed from common buckwheat was tested in other wild and cultivated *Fagopyrum* spp. (Joshi et al., 2006) which indicated that the transferability of common buckwheat EST markers decreased with an increase in genetic distance between species.

The presence of a genome library is useful for achieving advances in genomics and breeding of buckwheat. For example, several interesting and economically important genes can be isolated by positional cloning, such as the heterostylous self compatibility gene (Aii et al., 1998) or various stress resistance genes. A BAC library has been constructed from wild

buckwheat, *F. homotropicum* (Nagano et al., 2001b). This library contains 24,096 clones with an average insert size of 97 Kb. Another BAC library for *F. esculentum* has been constructed, which includes 1, 42,005 clones with an average insert size of ~76 kb (Yasui et al., 2008).

Transcriptome sequencing data has become an integral component of modern genetics, genomics and evolutionary biology. However, despite advances in the technologies of DNA sequencing, such data are lacking for many groups of living organisms, in particular, many plant taxa. 454 transcriptome sequencing and *de novo* assembly was performed for two congeneric flowering plant species, *F. esculentum* and *F. tataricum*. As a result, a large set of cDNA sequences that represent orthologs of known plant genes as well as potential new genes was generated (Logacheva et al., 2011). Chloroplasts often show uni-parental inheritance over generations, which provide valuable information on interspecies relationships. The complete chloroplast genome sequence of the wild ancestor of cultivated buckwheat, *F.esculentum* ssp. *ancestrale* has been made available by Logacheva et al. (2008).

2.3.1.) Genetic diversity analysis in *Fagopyrum* species

Genetic diversity of plant genetic resources for food and agriculture is a unique and irreplaceable source for further crop genetic improvement. Genetic diversity has been analyzed in buckwheat germplasm with different molecular markers such as RAPD (Yang et al., 2010), AFLP (Tsuji and Ohnishi 2001) and SSRs (Li et al., 2007). Sharma and Jana (2002a) studied species relationships in 14 *Fagopyrum spp* using RAPD markers which revealed that *F. tataricum* is closer to its wild ancestor *F. tataricum* ssp. *potanini* Batalin, closely followed by *F. giganteum*. High genetic diversity was found among natural populations and cultivated landraces of tartary buckwheat through RAPD and AFLP markers (Tsuji and Ohnishi 2001; Kump and Jovornik 2002; Sharma and Jana 2002b; Senthilkumaran et al., 2007). The inter and intra specific genetic

diversity in accessions of buckwheat growing in different regions of Indian Himalayas was also studied by using single seed protein SDS-PAGE analysis (Chrungoo and Anusuya 2004).

A phylogenetic tree, reconstructed from cpDNA sequences, demonstrated that *F. tataricum* (including both wild and cultivated forms), a close relative of *F. cymosum*, was completely included in the Tibet-Himalayan clade of *F. cymosum*. It was found that natural populations of *F. cymosum* maintained a high amount of genetic variations within the species, whereas *F. tataricum* possesses a fairly low level (Yamane et al., 2004). Zhang et al. (2007) showed that there was 86.5% polymorphism in 79 accessions of tartary buckwheat germplasm collected from china. High genetic variability was found among *F.esculentum* populations based on morphological and physiological characters (Iwata et al., 2005; Cepková et al., 2009). Genetic diversity in Japanese indigenous common buckwheat (*F. esculentum*) cultivars using AFLP and SSR markers investigated the relationships between the genetic diversity and agronomic traits (Iwata et al., 2005). The genetic diversity of 19 common and tartary buckwheat varieties including the tested varieties in Guizhou region during 1999-2010 and their parents were studied using 7 primers by means of RAPD accounting for 94.89% of polymorphism (Lin qiong 2011). However, none of the studies on characterization of genetic diversity with molecular markers has attempted to correlate diversity at DNA level with any of the desirable traits in buckwheat.

2.4) Rutin- a major flavonoid constituent of buckwheat

The flavonoid rutin (quercetin 3-O rutinoside), is a flavonol glycoside comprised of the flavonol quercetin and the disaccharide rutinose. Its name comes from the name of *Ruta graveolens*, a plant that also contains rutin. Rutin has an ability to increase intracellular ascorbic acid levels, decreases capillary permeability and fragility, scavenge oxidants and free radicals, inhibits destruction of bones as well as lowers the risk of heart diseases (Middleton et al., 1984; Kreft et

al., 1999; Saikia and Deka 2011). The use of rutin as medicinal agent has stimulated interest in this compound. Rutin is present in substantial amounts in various plant species such as *Viola tricolor* (3.36%), *Cappris spinosa* (0.28%), Apple (0.17%), *Lycopersicon esculentum* (0.002%-0.009%) and many more (McGregor and McKillican 1952; Attanassova and Bagdassarian 2009). Analysis of rutin content in leaves and flowers of 50 medicinal plants of rue, buckwheat, rose, sage, clove, rose etc showed that content was highest in leaves of rue (8.6%), followed by flowers (5.3%) and leaves (2.0%) of buckwheat, flowers of pansy (3.35%) and flowers of rose (1.0%) while others contained less than 0.05% of rutin content (Sofic et al., 2010). Musallam et al. (2012) also reported variation in rutin content in *Capris spinosa* with 2.76% in leaves, 1.8% in flower buds and 0.28% in fruits.

Buckwheat is one of the richest sources of rutin in seeds, flowers, leaves, stem and roots. Rutin has been identified in higher amounts in *F. cymosum* (0.01%), *F. esculentum* (0.01%) and *F.tataricum* (0.8-1.8%) (Fabjan et al., 2003). Rutin content in plant parts of these 3 species, was highest in the order of flower>leaf>seed>stem>root (Table 2.4) (Park et al., 2004). In addition, rutin content variation in sprouts of *F. esculentum* and *F. tataricum* was also observed with 5 times higher in *F. tataricum* over *F. esculentum* (Liu et al., 2008).

Table 2.4 Rutin content variation in different tissues of *Fagopyrum* species (Park et al., 2004)

	Rutin Content %				
Species	**Flower**	**Leaf**	**Stem**	**Root**	**Seed**
F. esculentum	0.37	0.12	0.017	0.01	0.023
F. tataricum	3.52	2.87	0.48	0.02	1.47
F. cymosum	1.58	0.92	0.017	-	0.45

2.5) Biosynthesis of flavonoids (rutin)

The precursors of the synthesis of most flavonoids are malonyl-CoA and p-coumaroyl-CoA, which are derived from carbohydrate metabolism and phenylpropanoid pathway, respectively (Forkmann and Heller 1999). The biosynthesis of flavonoids is initiated by the enzymatic step catalysed by chalcone synthase (CHS), resulting in the yellow coloured chalcone. In the majority of plants chalcones are not the end-products, but the pathway proceeds with several enzymatic steps to other classes of flavonoids, such as flavanones, dihydroflavonols and finally to the anthocyanins, the major watersoluble pigments in flowers and fruits. Other flavonoid classes (i.e. isoflavones, aurones, flavones, proanthocyanidins and flavonols) represent side branches of the flavonoid pathway and are derived from intermediates in anthocyanin formation (Figure 2.1).

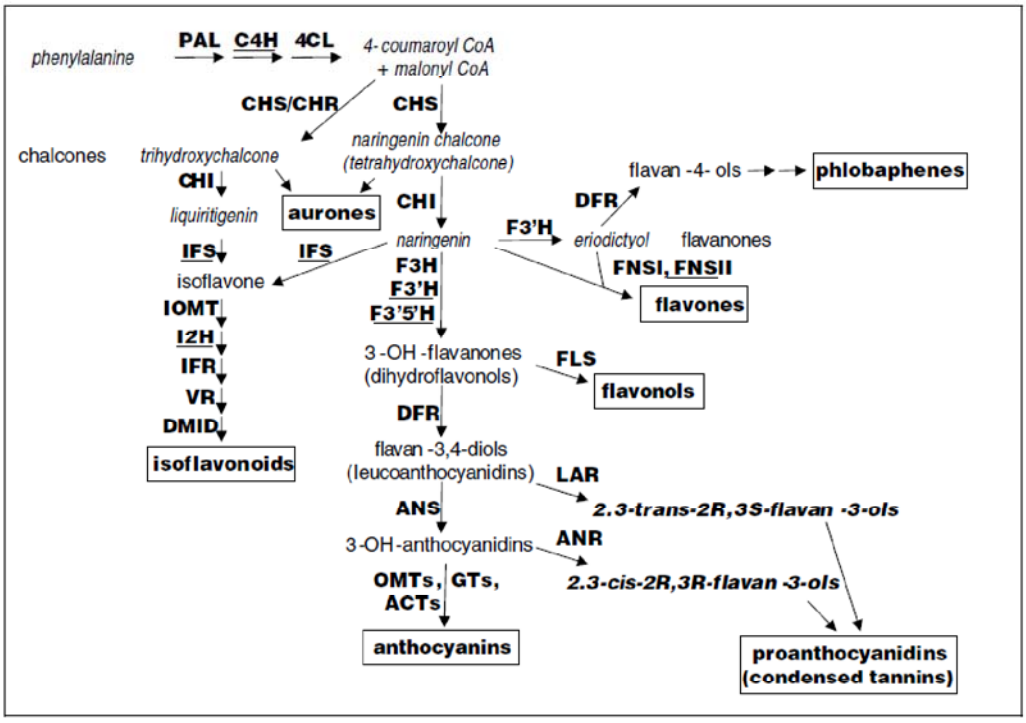

Figure 2.1 Schematic of the flavonoid pathway showing the enzymatic steps leading to the major classes of end products, flavonols, anthocyanins, proanthocyanidins, phlobaphenes, aurones, flavones, and isoflavonoids, which are identified with boxes. Enzymes are indicated with standard abbreviations (Grotewold 2006).

From the beginning of the pathway towards flavonols:

PAL catalyzes the first committed step of the phenylpropanoid pathway by catalyzing the conversion of the amino acid phenylalanine to *trans*-cinnamic acid (Figure 2.2). This reaction does not require any co-factor and the chemistry involves *trans*-elimination of ammonia from L-phenylalanine and the removal of pro-S hydrogen at C3. C4H introduces a hydroxyl group to C4 of cinnamic acid in the presence of molecular oxygen to yield *trans*-4-coumaric acid. C4H is a cytochrome P450 monooxygenase (CYP73A) that acts together with a NADPH-dependent cytochrome P450 reductase. C4H is encoded by a single copy gene in *Arabidopsis* and only one or two copies exist in other genomes studied to date (Mizutani et al., 1997). C4H was the first plant P450 to be functionally characterized, and is among the most extensively studied of the plant P450s (Chapple 1998). 4CL activates *p*-coumaric acid by the addition of coenzyme A to produce a 4-coumarate-CoA ester which is the preferred substrate of CHS. The flavonoid biosynthetic pathway starts with the condensation of one molecule of 4-coumaroyl-CoA and three molecules of malonyl-CoA, yielding naringenin chalcone. This reaction is carried out by the enzyme CHS. Chalcone is isomerised to a flavanone by the enzyme CHI. From these central intermediates, the pathway diverges into several side branches, each resulting in a different class of flavonoids. F3H catalyzes the stereospecific 3ß-hydroxylation of (2S)-flavanones to dihydroflavonols. For the biosynthesis of flavonols, F3'H converts the dihydroflavonol to dihydroquercrtin which then gets converted into flavonol quercetin via the activity of enzyme FLS. As the final step of flavonol biosynthesis, glucosyl/rhamnosyl transferases (GT) catalyses the transfer of glucose and rhamnose from UDP-glucose, UDP-rhamnose for the synthesis of flavonol glycoside (rutin).

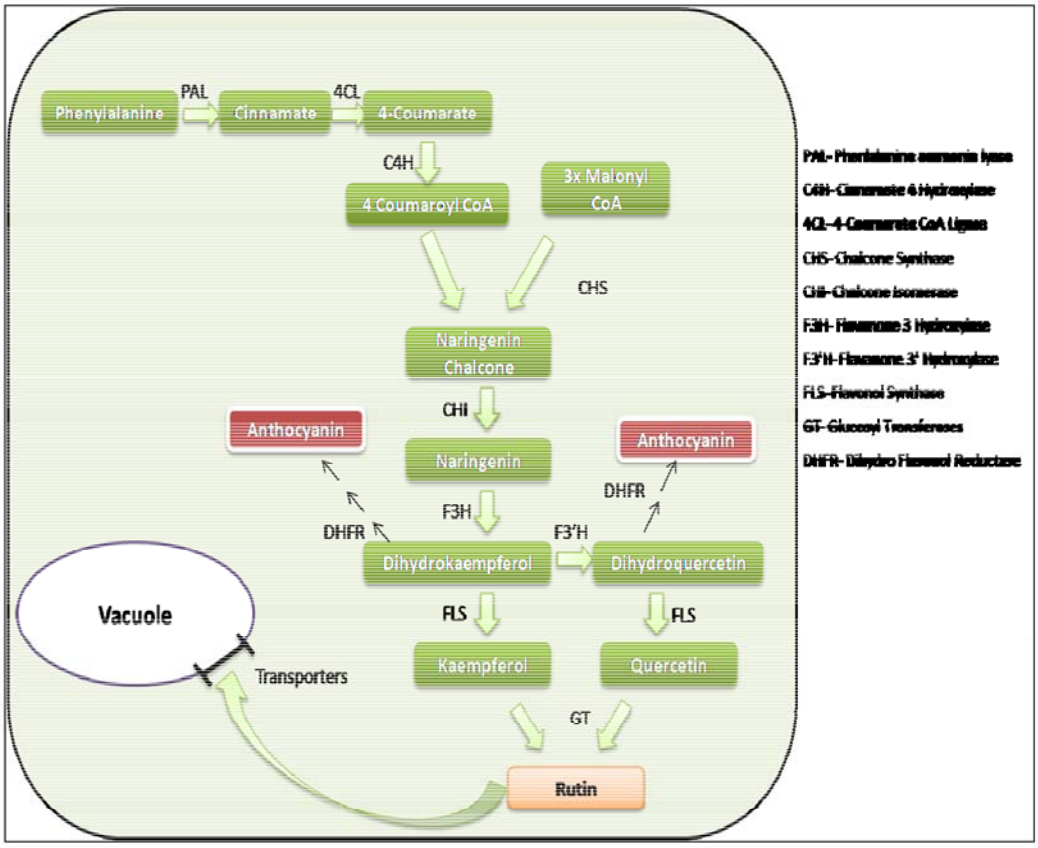

Figure 2.2 Rutin biosynthesis pathway

2.6) Factors affecting biosynthesis and accumulation of rutin in different plant species

The flavonoid biosynthetic pathway is under tight developmental control, and multiple environmental conditions of light and hormones affect the expression of flavonoid biosynthetic genes (Irani et al., 2003). Indeed, a decrease of flavonoid biosynthesis was observed when either endogenous (e.g. plant hormones), or exogenous factors (e.g. water and temperature stress, light, fertilizer, etc.) are limiting or excessive. UV-radiations stimulated the activity of enzymes of phenylpropanoid pathway and therefore, influenced rutin content in plants. The comparison of different UV-B treatments in buckwheat revealed that under ambient light conditions 97% more rutin content accumulated in leaves in comparison to leaves grown under reduced UV-B radiations (Kreft et al., 2002). Accumulation of flavonoids increased in leaves of ginger grown in

60% shade which revealed that flavonoids are light dependent and their biosynthetic rate is related to light intensity (Ghasemzadeh and Ghasemzadeh 2011). Under stress conditions like drought, salinity etc. rutin content increased in seedlings of *Dimorphandra mollis*, suggesting their role in protecting tissues against oxidative damage (Lucci and Mazzafera 2009). Environmental factors that increase plant vigour (e.g. excessive fertilizer) are reported to negatively influence flavonoid content in grape berry. Nitrogen and potassium at high levels caused the enhancement of vegetative growth, the delay of ripening and a decrease of color in grape berry (Braidot et al., 2008).

Jamonates play a central role in regulating the biosynthesis of many secondary metabolites including flavonoids. Methyl jasmonate showed an inhibitory effect on rutin content in Cucurbita and Buckwheat (Stoynova-Bakalova et al., 2009; Horbowicz et al., 2011). Methyl jasmonate decreased the contents of all the found cyanidin glycosides and its aglycone in hypocotyls of buckwheat seedlings (Horbowicz et al., 2011). Growth hormone, cytokinin also increased 3 folds of rutin accumulation in cucurbita (Stoynova-Bakalova et al., 2009). The plant growth regulator, naphthalene acetic acid also increases rutin content in *Ficus deltoidea* (Ong et al., 2011). Concentration of biomolecules also affects the rutin content in various plants. For example, the concentration of rutin was enhanced 2 folds in *Ficus deltoidea* by culture enriched in biomolecules glucose or fructose (Ong et al., 2011). Similarly the accumulation of rutin increases with increased in sucrose content in *Arabidopsis*, buckwheat seedlings along with the expression of flavonoid genes (Solfenic et al., 2006; Li et al., 2011). However, not much information is available on biosynthesis and accumulation of rutin vis-à-vis growth and development of different stages in a particular species.

2.7) Molecular basis of flavonoid contents in plants

Analysis of flavonoid biosynthesis has focused largely on gene expression, uncovering coordinate patterns of expression that are controlled by distinct sets of transcriptional regulators. The first major progress toward understanding the biochemistry and genetics of flavonoid metabolism came from studies of inheritance, starting with Mendel's use of flower color in pea to study the segregation of visible traits (Mendel 1865). The finding that purple-flowered progeny could be produced by crossing different white-flowered lines gave rise to the hypothesis that two genetic factors, *C* and *R*, were required for the production of red pigments in sweet pea. In recent years, much effort has been directed at elucidating the flavonoid biosynthetic pathway from a molecular genetics point of view. Mutants affecting flavonoid synthesis have been isolated in a variety of plant species based on alterations in flower and seed pigmentation. Maize, snapdragon (*Antirrhinum majus*), and petunia were established as the first major experimental models, and work in these species led to the isolation of many flavonoid structural and regulatory genes (Holton and Cornish 1995; Mol et al., 1998). *Arabidopsis* more recently has helped facilitate analysis of the regulation and sub-cellular organization of the flavonoid pathway. The initial collection of 12 *tt* mutants has been expanded to include 21 members (*tt1–19* plus *ttg1 and ttg2)*, through yellow or pale-brown seeds, and indirectly exhibiting reduced seed dormancy. Transposon and activator tagging have been used to isolate additional mutations in genes either directly or indirectly involved in flavonoid biosynthesis (Kubo et al., 1999; Borevitz et al., 2000). As a result most of the structural genes, as well as a number of regulatory genes, have now been correlated with specific mutant loci in *Arabidopsis*.

The flavonoids are differentially distributed in different tissues of various plant species. For example, leaves of larch has highest flavonone content in comparison to flowers and fruits,

in different growth stages of American elderberry rutin content varied with 0.67% in leaves, 0.55% in flowers, and 0.018% in green stems (Vvedenskaya and Vorsa 2004). This variation in content was attributed to the differential expression of structural and regulatory genes involved in flavonoid biosynthesis (Winkel-Shirley 2001).

2.7.1) Role of structural genes in controlling flavonoid (rutin) biosynthesis in plants

The structural genes have been used to engineer the flavonoid biosynthetic pathway in both model and crop plant species, not only from a fundamental perspective, but also in order to alter important agronomic traits, such as flower and fruit color, resistance to pests and nutritional value.

The first report of metabolic engineering of the flavonoid pathway structural genes in plants was published in 1987. Meyer et al. (1987) introduced the dihydroquercetin 4-reductase gene (A1) from maize into petunia, which enabled the production of pelargonidin in the transgenic plants and led to a change in flower color. Since then, metabolic engineering of the flavonoid pathway has primarily targeted floral colors in horticultural plants (Dixon and Steele 1999; Forkmann and Martens 2001). F3′H and F3′5′H were key enzymes in engineering blue flowers due their role in the biosynthesis of delphinidin (Katsumoto et al., 2007). Anthocyanin production was further increased by downregulating expression of flavonol synthase (FLS) (Davies et al., 2003). This study demonstrated that FLS was a key enzyme in the regulation of flux into different branches of flavonoid biosynthesis. It has been revealed that flavonoids were essential for pollen tube growth and fertilization. The suppression of CHS, the first enzyme in the flavonoid pathway, resulted in the reduction of flower pigmentation and inhibition of male sterility in *Petunia* (van der Meer et al., 1992). In tomato, RNAi mediated silencing of CHS resulted in production of parthenocarpic fruit displaying impaired pollen growth (Schijlen et al.,

2007). In fact, transgenic tomato plants expressing the petunia CHI gene displayed increased levels of fruit peel flavonols, mainly due to the accumulation of rutin, and a concomitant reduction of naringenin chalcone (Muir et al., 2001). Also in potato tubers, overexpression of CHS and DFR from petunia resulted in increase of anthocyanins. In transgenic tobacco plants overexpression of AtPAL2 showed two and five times increases of chlorogenic acid and rutin levels than the wild-type plants, respectively (Chang et al., 2009). Notable case studies involving metabolic improvements concerning increases in the biosynthesis of stilbenoids and flavonoids in some edible plant parts and food crop plants are listed in table 2.5.

2.7.2) Transcriptional regulation of flavonoid biosynthesis

Coordinate transcriptional control of biosynthetic genes has emerged as a major mechanism dictating the final levels of secondary metabolites in plant cells. This regulation is achieved by specific transcription factors. Regulation of structural gene expression appears tightly organized in a spatial and temporal way during plant development, and is orchestrated by a ternary complex involving transcription factors from the R2R3-MYB, basic helix–loop–helix (bHLH), and WD40 classes (Hichri et al., 2011). Several genes encoding transcription factors from these three families have been identified, however, many gaps remain in our understanding of the regulation of the biosynthetic pathways, especially about the respective roles of bHLH and WD40 proteins. Taken together all four factors that affect metabolic engineering-structural genes over-expression or silencing, transcription regulation, flux control and transporter over-expression have been applied in the manipulation of flavonoid biosynthetic pathway resulting in altered flavonoid content in plants (Hichri et al., 2011).

Transcription factors have also been exploited in the metabolic engineering of flavonoid and phenylpropanoid metabolism because: (i) transcription factors typically control the

expression of multiple genes encoding enzymes in a given pathway, allowing efficient manipulation of multienzyme pathways, (ii) ectopic expression of specific transcription factors can be used as a tool for redirecting metabolic differentiation of the cells, (iii) pathway-specific transcription factors could be used to modulate the production of specific secondary metabolites (Table 2.6). In tomato introduction and over-expression of maize transcription factors Lc and C1 lead to 60% increase of kaempferol formation (Bovy et al., 2007). In contrast to maize, where LC and C1 regulate all genes of the of the pathway from CHS until 3GT, it has been shown that in dicots such as *Petunia* and *Antirrhinum* distinct sets of MYB/MYC transcription factors are responsible for regulating the early part (CHS up to F3H) or the late part (DFR to 3GT) of the pathway (Bovy et al., 2007). The *Antirrhinum Delila* gene (DEL), a MYC (bHLH) homologue, is required for pigmentation of the flower tube (Mooney et al., 1995). Over-expression of *Delila* in tobacco and tomato resulted in enhanced pigmentation of vegetative tissues in tomato whereas only the flowers were affected in tobacco by over-expression of F3H, CHS and DFR. A 10-fold increase of DFR mRNA levels was observed in tomato and a 4-fold increase in tobacco when *DEL* was over-expressed (Mooney et al., 1995). A Myb transcription factor gene was identified by activation tagging in a tomato line accumulating anthocyanins due to up-regulation of anthocyanin pathway genes (Mathews et al., 2003). This gene encoded the *ANT1* protein, which showed strong similarity with the *Petunia AN2* (MYB) protein. Fruit specific RNAi-mediated suppression of the tomato regulatory gene *DET1*, which normally represses light controlled signalling pathways, resulted in a "high pigment" fruit phenotype. In addition to enhanced carotenoid levels, these *DET1*-RNAi fruits contained up to 3.5 fold increase in flavonoid content (Davuluri et al., 2005).

Besides positively regulating the activity of flavonoid pathway, some TFs also negatively regulate the flavonoid biosynthesis. Over-expression of the *FaMYB1* transcription factor isolated from red strawberry fruits resulted in suppression of anthocyanins as well as flavonol accumulation in tobacco (Aharoni et al., 2001). Also two MYB transcription factors from *Antirrhinum*, *AmMYB308* and *AmMYB330* were shown to have repressing effects on genes involved in phenylpropanoid biosynthesis when expressed in tobacco (Tamagnone et al., 1998). The overexpression of maize *ZmMYB31* greatly reduces the synthesis of lignin and represses the accumulation of sinapoylmalate, redirecting the metabolic flux toward flavonoid biosynthesis (Fornale et al., 2010). However, most of the transcription factors characterized to date belong to anthocyanin pathway, only few reports were available for flavonol pathway (Hichri et al., 2011).

However, fewer reports are available on the transcriptional regulation of rutin biosynthesis in different plant species. A *MYB* transcription factor, *AtMYB12* is a flavonol-specific activator of flavonoid biosynthesis and a transcriptional regulator of chalcone synthase (CHS) and flavonol synthase (FLS), which were indispensible for the biosynthesis of flavonols in *Arabidopsis* (Mehrtens et al., 2005). *AtMYB12* regulate the phenylpropanoid pathway by increasing the accumulation of rutin in expressing transgenic tobacco lines. High accumulation of rutin in *AtMYB12*-expressing tobacco lines provided resistance against *Spodoptera litura* and *Helicoverpa armigera* insects (Misra et al., 2010). In addition, silencing of tomato *SlMYB12* gene leads to pink tomatoes due to decrease in naringenin chalcone and rutin accumulation than red colored phenotype (Ballester et al., 2009). *AtMYB12* also resulted in significant enhancement of rutin levels in transgenic buckwheat hairy roots by up-regulation of flavonoid biosynthesis genes (Park et al., 2011b). Another transcription factor PAP1/AtMYB75 from *Arabidopsis thaliana* lead to higher accumulation of flavonoids like anthocyanins, rutin, isoquercitin,

kaempferol-glucoside etc in transgenic plants of *Humulus lupus* compared to wild-type plants (Gatica-Arias 2012). Transcription factors contributing to the *WRKY* family lead to induction of genes involved in the central phenylpropanoid pathway with higher rutin accumulation in *Medicago* transgenics (Naoumkina et al., 2008).

Table 2.5 Metabolic engineering of flavonoid biosynthetic pathway in different plant species

Metabolic target	Plant gene donors (Target gene(s))	Engineered plant	Metabolic effect	References
Apigenin	*Saussurea medusa* (CHI)	*S. involucrata*	Overproduction of apigenin through CHI over-expression	Li et al. (2006)
Kaempferol glycoside, quercetin glycoside	*P. hybrida* (CHI)	*L. esculentum*	Production of increased level of kaempferol and quercetin glycoside	Muir et al. (2001)
Naringenin, kaempferol, rutin, quercetin	*L. esculentum* (CHS, CHI, FLS)	*L. esculentum*	Increase in total flavonoid content	Verhoeyen et al. (2002)
Chalcones, flavones, flavonols	*P. hybrida* (CHS, CHI), *M. sativa* (CHR), Gerbera (FNS II)	*L. esculentum*	Novel flavonoid production in non-producing fruit	Schijlen et al. (2007)
Anthocyanins	*P. hybrida* (CHS, CHI, DFR)	*S. tuberosum*	Altered anthocyanin level in potato	Stobiecki et al. (2003)
Anthocyanins	*S. sogarandinum* (A5GT)	*S. tuberosum*	Increase in resistance against *Erwinia*	Lorenc-Kukula et al. (2005)
Quercetin, proanthocyanins, anthocyanins	*O. sativa* (ANS)	*O.sativa*	Accumulates mixture of flavonoids	Reddy et al. (2007)
Lignans, total phenolics	*P. hybrida* (CHS, CHI, DFR)	*Linum usilatissinrum*	Increase in total phenolic antioxidant level	Lorenc-Kukula et al. (2005)
Kaempferol, Phenolic acids	*P. hybrida* (CHS, CHI, DFR)	*Linum usilatissinrum*	Accumulation of several flavonoids, phenolic acids and lignans	Zuk et al. (2011)

Table 2.6 TFs involved in regulation of flavonoid biosynthesis pathway

Species	Protein	Function(s)	References
bHLH			
Zea mays (maize)	ZmB ZmR ZmLc	Regulation of the anthocyanin pathway	Chandler et al. (1989) Ludwig et al. (1989) Goff et al. (1990)
	Zmln1	Repression of flavonoid gene expression in maize aleurone	Burr et al. (1996)
Arabidopsis thaliana	AtTT8	Control of proanthocyanidins and anthocyanins in seed and seedlings	Nesi et al. (2000)
	GL3/EGL3	Induction of anthocyanin accumulation	Bernhardt et al. (2003)
Vitis vinifera	VvMYC1	Promotion of anthocyanin accumulation	Hichri et al. (2010)
Antirrhinum majus	Delila	Control of anthocyanin biosynthesis in *L. esculentum* flowers	Martin et al. (1991)
Petunia hybrida	PhAN1	Control of anthocyanin biosynthesis in flowers	Quattrocchio et al.(1993)
L. esculentum	MYC-RP, MYC- GP	Increase in anthocyanin content	Gong et al. (1999)
MYB			
Zea mays	ZmC1 ZmPl1 ZmP1	Control of flavonoid biosynthesis in kernels	Paz-Ares et al. (1987) Chandler et al. (1989) Goff et al. (1990)
	P1	Increase anthocyanin and flavone level	Koes et al. (1994)
Arabidopsis thaliana	AtMYBL2	Repression of anthocyanin biosynthesis	Dubos et al. (2008)
	AtMYB12	Increase anthocyanin and flavone level	Park et al. (2011b)
	TT2, PAP1	Ectopic accumulation of proanthocyanidins	Sharma and Dixon (2005)
	AtMYB60	Inhibits anthocyanin synthesis in lettuce	Park et al. (2008)
Fragaria	FaMYB1	Inhibits anthocyanin synthesis	Aharoni et al. (2001)
Vitis vinifera	VvMYBPA1 VvMYBPA2	Induction of proanthocyanidin synthesis	Bogs et al. (2005)
	VvMYB5a	Regulation of phenylpropanoid accumulation	Deluc et al. (2008)
L. esculentum	ANT1	Increase anthocyanin content	Mathews et al. (2003)
Antirrinum majus	AmMYB308 AmMYB330	Control of phenylpropanoid biosynthesis	Tamagnone et al. (1998)
WD40			
Zea mays	ZmPAC1	Regulation of anthocyanin pathway in seed alurone	Carey et al. (2004)
Arabidopsis thaliana	AtTTG1	Control of flavonoid pathway	Walker et al.(1999)
Vitis vinifera	WDR1	Contributes to the accumutation of anthocyanins	Matus et al. (2010)

2.8) Transport of Flavonoids

The accurate delivery and sequestration of chemically reactive and potentially toxic metabolites pose a significant challenge for plant cells, which can simultaneously accumulate hundreds of different phytochemicals, derived from both primary and secondary metabolism. The proper sub-cellular localization of flavonoids is crucial for fullfiling their functions in plant cell. However, the transport mechanisms involved in trafficking of most primary and secondary metabolites are still poorly understood. Two major hypotheses have been proposed for flavonoid transport: membrane vesicle-mediated transport and membrane transporter-mediated transport (Figure 2.3).

Sub-cellular compartmentalization of flavonoids

Anthocyanins, proanthocyanidins (also called condensed tannins), and flavonols are three major subclasses of flavonoid compounds. Anthocyanins do not show their brilliant colors until they are accumulated in the acidic vacuoles (Grotewold 2006). In cells, anthocyanin pigments are synthesized at the cytoplasmic surface of the endoplasmic reticulum, and are then transported and finally accumulated inside the vacuole. The oxidation of proanthocyanidins hardens the seed coat, which induces moderate dormancy in the seeds and limits the detrimental effects of physical and biological attacks. Thus, the proper sub-cellular localization of these flavonoids is crucial for fulfilling their functions in plant cells. Some flavonols have a protective role as UV-B filters, and they also function as co-pigments for anthocyanins in specific tissues. To fulfill these functions, such flavonols are assumed to accumulate in the vacuole (Koes et al., 1994; Mol et al., 1998).

2.8.1) Cellular transport of flavonoids

The probable biosynthetic sites of flavonoids 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. It has been suggested that flavonoid moieties, depending on their different substituting groups (acyl, glycosyl and/or methoxyl), are driven to their accumulation sites by a complex vesicle trafficking system involving the Golgi apparatus (Grotewold 2006). Such a vesicle network requires the participation of specific transporters able to upload flavonoids inside vesicles. Anthocyanoplasts were first assumed to be transport vesicles or sites of anthocyanin biosynthesis (Grotewold 2004). These cytoplasmic anthocyanin bodies are covered by a membrane and originate from a large number of smaller vesicle-like structures that gradually fuse together. Similar cellular structures, anthocyanic vacuolar inclusions (AVIs), are present in the vacuoles of many species and also show dynamic movement (Markham et al., 2000). Although associated with specific proteins and membranous substances, AVIs are not surrounded by a membrane (Markham et al., 2000), and are more likely to be storage complexes than to be involved in anthocyanin transport. In addition, anthocyanin-containing vesicle-like structures can co-localize with protein storage vacuoles (PSVs) and transport anthocyanins in a trans-Golgi network (TGN)-independent ER-to-PVC vesicle trafficking pathway.

Different types of transporters participating in the vacuolar uptake of flavonoids are described below:

Proton-Dependent Transporters: Vacuoles have various physiological functions in plant development and differentiation. One of their functions is to act as a storage site for various secondary metabolites including flavonoids. In plants, the acidic conditions of the vacuolar lumen are established mainly by two tonoplast-localized proton pumps: the v- ATPase and the v-PPase (Maeshima 2001). The pH gradient between the cytosol and the vacuole provides the potential energy needed to take up substrates into the vacuole. Hopp and Seitz (1987)

investigated the uptake efficiency of radiolabeled anthocyanins into isolated carrot vacuoles, and suggested that vacuolar uptake of flavonoids was dependent on the energy provided by the proton gradient. Klein et al. (1996) suggested the existence of a proton antiporter in barley for the uptake of radiolabeled flavones glucosides *in vitro*.

ABC-Type Transporters: The *Arabidopsis*, rice (Oryza sativa), and *M. truncatula* genomes each have >120 putative ABC transporters, but only a few have been characterized. The glutathione conjugates are recognized and sequestered to the vacuoles or exported to the cell wall by ATP-dependent, protongradient- independent transporters, named ATP-binding cassette (ABC)-type transporters (Theodoulou 2000; Kang et al., 2011). No other types of transporters are known to mediate compartmentalization of the glutathione conjugates. The involvement of an ABC-type transporter in flavonoid accumulation was first suggested by Marrs et al. (1995). Flavone glucuronides of rye also were taken up by the vacuolar membrane *in vitro* through an ABC-type transporter. *Arabidopsis* MRP-type ABC transporters (AtMRP1 and AtMRP2) mediate the vacuolar uptake of anthocyanin–glutathione conjugates in a heterologous host (yeast) (Lu et al., 1998; Kang et al., 2011). Suppression of the maize tonoplast-localized ABC-type transporter ZmMRP3 results in reduced anthocyanin accumulation (Goodman et al., 2004).

MATE-Type Transporters: Multidrug and toxic compound extrusion (MATE) family transporters use H+/Na+ gradients across membranes as a force to drive waste or toxic compounds out of the cytoplasm. The *Arabidopsis* genome has more than 50 genes encoding putative MATE-type proteins, and a few mutants with defective MATE-type genes have been isolated. Proanthocyanidins deficiency in seeds of the *Arabidopsis* tt12 mutant is caused by loss of function of a MATE transporter, TT12, which mediates vacuolar sequestration of cyanidin 3-

O-glucoside and is localized to the tonoplast (Zhao and Dixon 2009). It is now known that TT12 and its ortholog MATE1 from *M. truncatula* expressed in yeast preferentially transport epicatechin 3'-O-glucoside, which is proposed to be a precursor for the biosynthesis of PAs (Zhao and Dixon 2009; Zhao et al., 2011). Recently, two MATE-type transporters from grapevine (*Vitis vinifera*), AM1and AM3, were characterized as vacuolar transporters of acylated anthocyanins (Gomez et al., 2009), and the tomato (*Solanum lycopersicon*) MATE transporter LaMTP77 might have a similar function (Mathews et al., 2003). A genetic loss-of-function mutant of MATE2, a transporter of anthocyanins, led to the disappearance of leaf anthocyanin pigments as a result of drastic decreases in the levels of various flavonoids, presumably via redirection of metabolic flux from anthocyanin storage (Zhao et al., 2011).

Most of the studies on role of transporters in transporting flavonoids/anthocyanins are for color pigments which are visible and easy to record phenotypes in contrast to several other flavonoids which require their quantification in tissues through chromatographic techniques.

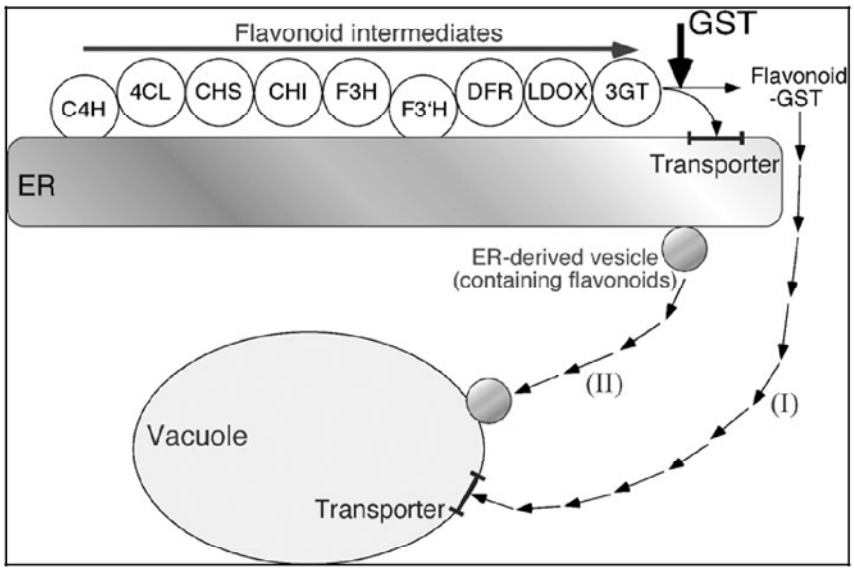

Figure 2.3 Proposed flavonoid transport mechanisms (Grotewold 2006).

The complete review of literature therefore, highlights the following gaps in our understanding on molecular basis of rutin biosynthesis in general and buckwheat in particular:

1. None of the studies on characterization of genetic diversity with molecular markers has attempted to correlate the diversity at DNA level with any of the desirable traits in buckwheat.
2. No information is available on biosynthesis and accumulation of rutin vis-à-vis growth and development of different stages in a particular species.
3. Fewer reports are available on transcriptional regulation of rutin biosynthesis.
4. Most of the studies on regulation of flavonoid biosynthesis transporters are for colored pigments in contrast to other flavonoids which require their quantification.
5. No genes/alleles have been identified which contribute to higher level of flavonoid content in a particular species.

Chapter-III

MATERIALS AND METHODS

The present investigation was carried out in the Jaypee University of Information Technology, Waknaghat, Himachal Pradesh. The material used and the methodologies adopted to achieve objectives of the investigation are described here under:

Plant Material

To accomplish the designed experiments, plant material was collected from:

Source: National Bureau of Plant Genetic Resources (NBPGR), Regional Research Station, Phagli, Shimla (H.P.)

Sample collected: Mature Seeds of *Fagopyrum* accessions (Figure 3.1)

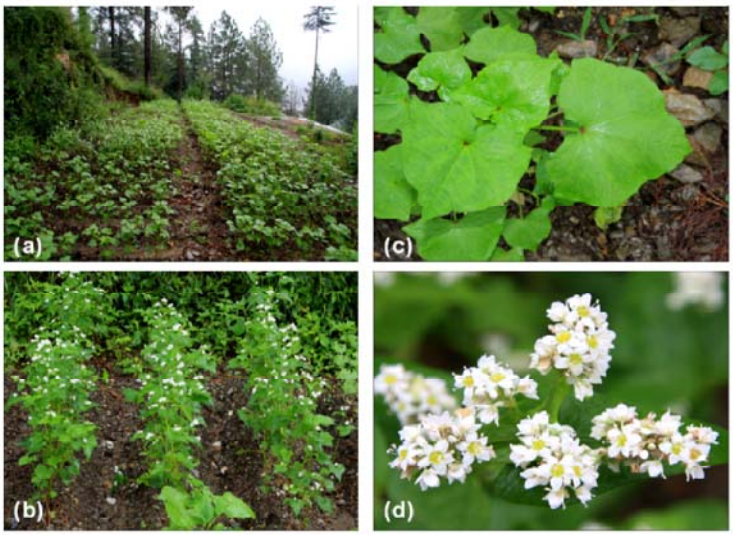

Figure 3.1 Field planting of high rutin content accessions of *F. tataricum*: (a) Accession in replications (b) Early flowering accession (35 Days) (c) Leaves ready for sampling (d) Buckwheat inflorescence

3.1.) Estimation of rutin content

3.1.1) Preparation of seed extract

Seeds were dried in a hot air oven for ≈48hr at 60^0C with regular monitoring of their weight and then homogenized at room temperature by using a grinder followed by pestle and mortar. The homogenized material was then sieved through mesh, 100mg of which was extracted in 80% methanol and incubated for 2 hrs. at 60^0C. Samples were centrifuged and filtered through 0.22 µm filters and quantified for rutin content by RP-HPLC.

3.1.2) HPLC analysis

The extract in 80% methanol was analyzed with Waters 515 HPLC system equipped with a model 515 solvent pump, a 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 x 2.6 mm, 3 µm) was used throughout this study. Flavonoids were detected at 350 nm. The mobile phase was acetonitrile and 0.04% TFA in a linear gradient for 35 minutes 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, Figure 3.2). Quantification was performed with the linear calibration curves of standard compounds. The significant differences between rutin contents were statistically analyzed.

High rutin content accessions were planted at the experimental farm of NBPGR, Phagli, Shimla (H.P.) for 2 years (2007-2009) and rutin content variation was validated through RP-HPLC (Figure 3.2). Data for rutin content analysis in high rutin content accessions was subjected to one way analysis of variance (ANOVA) to test significance of variation using MINITAB-14 at significant level of $P \leq 0.05$.

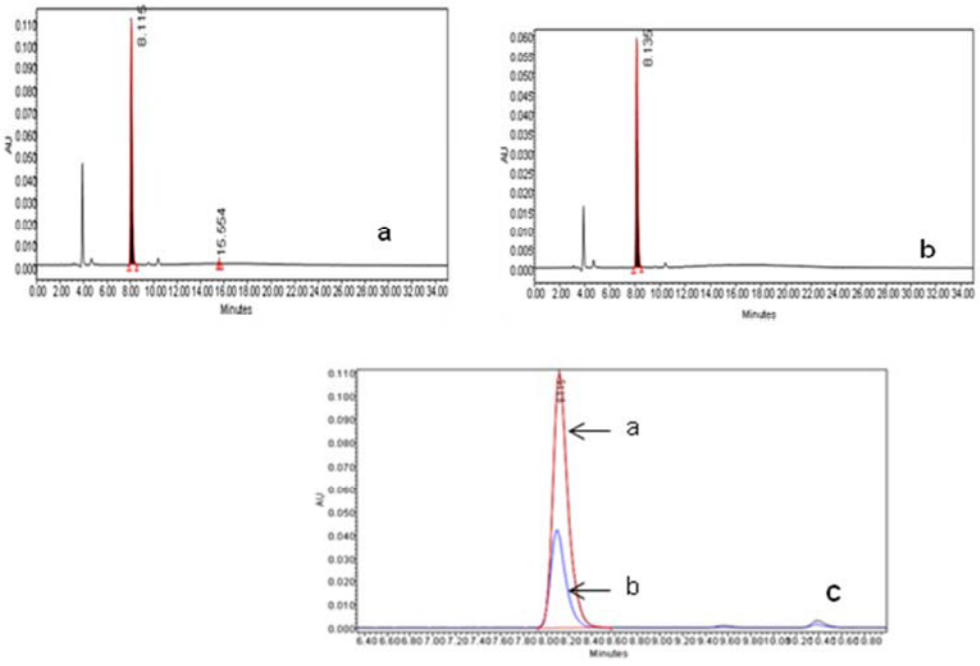

Figure 3.2 HPLC Chromatograms of rutin content analysis at stage S3 (a) Tartary buckwheat with initial sample weight of 0.48 gm (b) Common buckwheat accession with initial sample wt. of 0.85gm (c) Comparison of A & B chromatograms

3.2.) AFLP fingerprinting of tartary buckwheat accessions (*Fagopyrum tataricum*) displaying rutin content variation

In light of the economic importance of buckwheat as well as existence of enormous accessions of *Fagopyrum* species in the Himalayan regions of India, the characterization of tartary buckwheat for rutin content variation vis-à-vis DNA fingerprinting was undertaken. High and low rutin content accessions were selected to perform AFLP fingerprinting.

3.2.1) Isolation of genomic DNA

Genomic DNA of selected accessions of *F. tataricum* was isolated from leaf samples by following the protocol of Murray and Thompsan with little modifications (1980). The quality of

DNA was checked by 1% (w/v) ethidium bromide-stained agarose gel and from the absorbance spectrum at wavelengths 260nm and 280nm.

3.2.2) AFLP analysis

Amplified fragment length polymorphism analysis was performed based on the techniques of Vos et al. (1995) with some modifications. Genomic DNA (400 ng) was restricted with *Eco*RI and *Mse*I and ligated to standard adapters. The adapter-ligated DNA served as a template for pre-amplification, with PCR parameters of 30 cycles of 30 s at 94^{0}C, 60 s at 56^{0}C, and 60 s at 72^{0}C. The diluted (30-fold), amplified products were used as the template for selective amplification. The selective amplification was carried out using 21 selective primer combinations of *Eco*RI and *Mse*I (see Table 3.1) in a total volume of 20µl. The first selective amplification cycle consisted of 30 s at 94^{0}C, 30 s at 65^{0}C, and 60 s at 72^{0}C. The annealing temperature was lowered by 0.7^{0}C per cycle during the next 12 cycles, followed by 23 cycles at 94^{0}C for 30 s, 56^{0}C for 30 s, and 72^{0}C for 60 s. All PCR reactions were carried out in a Applied biosystem model no-9902 Veriti thermal cycler. To each PCR product was added 7.5 µl of formamide dye (98% formamide, 10 mM EDTA, 0.005% xylene cyanol FF, and 0.005% bromophenol blue), and 7 µl of each sample was loaded onto a pre-warmed 6% polyacrylamide gel using 1X Tris–borate–EDTA (0.05 M Tris, 0.05 M boric acid, 1mM, EDTA pH 8.0) electrophoresis buffer and run for 2.5 h at 65 W. The gels were silver-stained using silver staining kit (Promega cat. #Q4132, Madison, WI), following the manufacturer's instructions.

Table3.1. AFLP primer and adapter sequences

Adapters	**5′-Sequence-3′**
*Eco*RI forward adapter	CTC GTA TAC TGC GTA CC
*Eco*RI reverse adapter	AAT TGG TAC GCA GTA
*Mse*I forward adapter	GAC GAT GAG TCC TGA G
*Mse*I reverse adapter	TAC TCA GGA CTC ATC
Pre-selective primers	
*Eco*RI (00)	TAC TGC GTA CCA ATT C
*Mse*I (00)	GAC GAT GAG TCC TGA GTA A
Selective primers	
*Eco*RI(G)	TAC TGC GTA CCA ATT CG
*Eco*RI (C)	TAC TGC GTA CCA ATT CC
*Eco*RI(A)	TAC TGC GTA CCA ATT CA
*Eco*RI (AA)	TAC TGC GTA CCA ATT CAA
*Eco*RI (GA)	TAC TGC GTA CCA ATT CGA
*Eco*RI (AC)	TAC TGC GTA CCA ATT CAC
*Eco*RI (TA)	TAC TGC GTA CCA ATT C TA
*Eco*RI (TAG)	TAC TGC GTA CCA ATT CTA G
*Eco*RI (TGG)	TAC TGC GTA CCA ATT CTG G
*Eco*RI (TCC)	TAC TGC GTA CCA ATT CTC C
*Mse*I (C)	GAC GAT GAG TCC TGA GTA AC
*Mse*I (G)	GAC GAT GAG TCC TGA GTA AG
*Mse*I (A)	GAC GAT GAG TCC TGA GTA AA
*Mse*I (AC)	GAC GAT GAG TCC TGA GTA AAC
*Mse*I (GA)	GAC GAT GAG TCC TGA GTA AGA
*Mse*I (AA)	GAC GAT GAG TCC TGA GTA AAA
*Mse*I (CT)	GAC GAT GAG TCC TGA GTA ACT
*Mse*I (TTC)	GAC GAT GAG TCC TGA GTA ATT C
*Mse*I (TGA)	GAC GAT GAG TCC TGA GTA ATG A
*Mse*I (TCC)	GAC GAT GAG TCC TGA GTA ATC C
*Mse*I (CAT)	GAC GAT GAG TCC TGA GTA ACA T
*Mse*I (AAG)	GAC GAT GAG TCC TGA GTA AAA G

3.2.3) Gel Scoring and Data Analysis

For the genetic relationship studies, only distinct, reproducible, well-resolved AFLP fragments in size range of 100-500 bp were scored using software AlphaImager® EP as present (1) or absent (0), and a binary data matrix was constructed based on band scores. Bands of similar size and

intensity were assumed to be homologous. The percent polymorphism (PP) was calculated as PP = (P/N) X 100%; where P is the number of polymorphic bands and N is the total number of amplified bands. For the genetic relationship studies, only distinct, reproducible, well-resolved AFLP fragments in the size range of 100-500 bp were scored using software AlphaImager® EP as present (1) or absent (0), and a binary data matrix was constructed based on band scores. The PIC value for each AFLP primer combination was calculated based on Roldan-Ruiz et al. (2000) as PICi = 2fi(1 - fi); where PICi is the PIC of marker i, fi is the frequency of the marker bands which were present, and 1 - fi is the frequency of marker bands which were absent. One thousand bootstrapped replicate matrices of pairwise Fst among populations were calculated in AFLP-SURV. The results were used as inputs for computing Neighbor-Joining (NJ) dendrograms, using the DARwin (V5) software package. An extended majority-rule consensus tree was produced using CONSENSE a module for STRUCTURE v2.2 (Falush et al., 2007), adapted for dominant markers and used to assign an individual's probability of belonging to a homogeneous cluster (K populations) without prior population information. The correlated allele frequencies and admixed model were applied with a burn-in period of 100,000 and 1,000,000 MCMC replicates after burn-in. The range of clusters (K) was predefined from 1 to 10. From K=1 to K=10, 5 runs were performed. The Pr (X|K) (or ''LnP(D)'') can be used as an indication of the most likely number of groups, and it usually plateaus or increases slightly after the ''right K'' is reached (Evanno et al., 2005). Therefore, the height of the modal value of the ΔK distribution was calculated to detect the true K (Evanno et al., 2005) using Structure Harvester v 0.6.8 (Pritchard et al., 2000; http://taylor0.biology.ucla.edu/struct_harvest/). The similarity coefficient of pairwise runs for each value of K was also calculated in Structure 2(1).2- sum to control the stability of the results (Nordborg et al., 2005). POPGENE32 software was used to calculate

Nei's unbiased genetic distance between the different populations using all markers. Population diversity (Hs) and total gene diversity (Ht) were calculated by POPGENE software. Genetic diversity within and among populations was measured by the percentage of polymorphic bands (PPB). Estimate of gene flow (Nm) was calculated by the gene differentiation (Gst) using (0.25(1-Gst)/Gst).The partitioning of variation at different levels was calculated by Analysis of Molecular Variance (AMOVA) in GenAlEx using 999 permutations (Excoffier et al., 1992).

3.3) Selection of different growth stages of *Fagopyrum species*

On the basis of the rutin content estimation in 195 accessions of *F. tataricum*, three accessions, one each of tartary buckwheat, rice tartary buckwheat (easy de-hulling) and common buckwheat were chosen for further experiments for understanding molecular biology of rutin biosynthesis.

Rutin content analysis in different developmental stages of both the species (*F. tataricum* and *F. esculentum*) was done as per earlier given HPLC protocol so as to identify growth stage(s) with differential biosynthesis and accumulation of rutin. Nine different growth stages were considered right from the seed germination to mature seed formation (Figure 3.3). Seeds of three accessions, IC-14889, IC-329457 and IC-540858 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 $\mu molm^{-2}s^{-1}$), temperature (25±2°C), humidity (≈ 70%), and photoperiod of 14hrs day / 10hrs night. Samples of different growth stages (Figure 3.3) 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. For estimation of rutin content fresh weight samples of seeds, leaves and flowers were taken.

	Growth stage	Sample	Days after germination
Stage 1	Emergence	Whole plant	4
Stage 2	First pair of leaf formation	Whole plant	8
Stage 3	Bud show & leaf growth	Recently mature leaf (second)	15
Stage 4	Vegetative growth & leaf growth	Mature leaf	20
Stage 5	Flowering & mature leaves	Mature leaf	30
Stage 6	Peak flowering	Inflorescence	40
Stage 7	Seed formation started	Immature seeds	52
Stage 8	Seeds are in the milk or dough stage	Immature seeds	62
Stage 9	filled seeds are brown (Leaves have a yellowish cast)	Mature brown seeds	75

Figure 3.3 Different growth stages of *Fagopyrum* species selected for rutin content variation

3.4) Comparative genomics to clone flavonoid (rutin) biosynthesis genes in *Fagopyrum* species

The nucleotide and protein sequences of flavonoid biosynthesis genes were retrieved for different plant species from the Genbank (http://www.ncbi.nlm.nih.gov/genbank/) and then 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, yet short patches of conserved sequences were identified. Primer pairs were designed from the conserved regions of gene sequences (Figure 3.4, Table 3.2) and tested on genomic DNA and cDNA of *Fagopyrum* species.

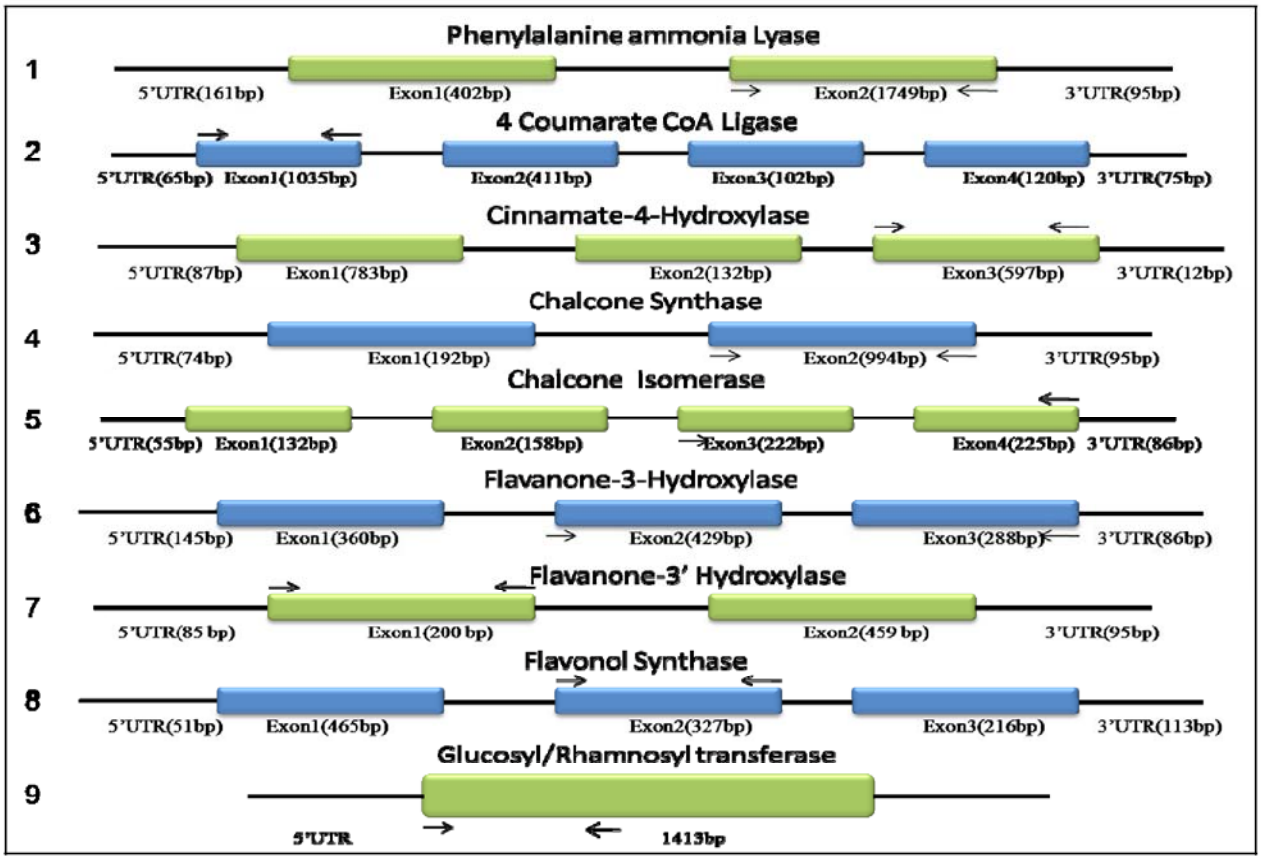

Figure 3.4 Gene structures of flavonoid biosynthetic pathway genes in *Arabidopsis thaliana*; Arrows represent the position of forward and reverse primers in the conserved sequence regions

Table 3.2 Primer sequences used to clone flavonoid biosynthetic (rutin) pathway gene sequences

Gene Name	Forward Primer Sequence (5'-3')	Reverse Primer Sequence (5'3')
PAL	GTGAAAGCGAGTAGTGATTGGGTG	GTTCACGTCTTGGTTGTGTTGCTC
C4H	TGGTCGATCGAGTGGGGAATTGC	TCATGAAGGTTCATGTGTGGGAC
4CL	CAGGCCTGACGATGTTGTGGCA	TCGCCATCTACTTGTTGAGCCA
CHS	CTCGAAGAACATCGAGAAGAGTC	CTTCTTCCTCATCTCATCCAAGAT
CHI	TCTCCGCTAAGTGGAAAGGCAA	CAAGAACTGCCTCACCCAACAA
F3H	ACTTTAGGACTCAAGCGCCACAC	GATCAGCATTCTTGAACCTCCCA
F3'H	TCCATGCTCGGTTTGACGCTTTCC	GGCGTTTTTGGGGATGTGGTAGC
FLS	TTCCACAGGGTTTGGCCTCC	CTCGATCTGGTCCCCGATGTGG
GT	GAGTGGAAAGAAAAGAAGAACGTC	GGGTATTGTTGGATTGGATAAGAG

For the isolation of genes in buckwheat, total genomic DNA was isolated from leaves of *Fagopyrum* species according to a modified CTAB-based procedure (Murray and Thompsan 1980). Also, total RNA was isolated from *Fagopyrum* samples of different growth stages by

using Raflex RNA isolation kit (GeNei^{TM}) 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 260nm and 280nm.

The cDNA was prepared from 5µg of RNA free from DNA (RNA was treated with 2U of DNase I), reverse transcribed by using M-MuLV reverse transcriptase (GeNei^{TM}) with oligo-dT primer. For amplification, PCR reactions for all the primer pairs were performed in 25 µl reaction volume. The PCR was performed on 30 ng genomic DNA and cDNA separately with varying amounts 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 sec, annealing temperature (63–55°C) for 45 sec, 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 6X gel loading dye (0.2% bromophenol blue, 0.2% xylene cyanol dye and 30% glycerol in TE Buffer) and electrophoresed in a 2% agarose gel prepared in 0.5X Tris borate-EDTA (TBE) buffer. The gels were analyzed using the gel documentation system AlphaImager EP (Alpha Innotech Corp., USA). The amplified genes (PCR products) were cloned in pGEMT vector (Promega) and sequenced. The identities of sequence similarities were calculated by Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST). Primer pairs for expression analysis through qRT-PCR were designed from confirmed sequences (Table 3.3).

Table 3.3 Primer sequences used in qRT-PCR analysis of rutin biosynthesis genes in *Fagopyrum* species

Gene Name	Forward Primer Sequence (5'-3')	Reverse Primer Sequence (5'3')
PAL	GCCTTGTAGGAGAGTGTTGATTCTG	AAGGAACTCATCAGATTTCTCAACG
C4H	GGTGGTTTACTAGCTCGCCTAATG	GTTACAGAAGGGTGAGATCAACGAG
4CL	GCATTCAACTTGTCCATGTTATC	TTATTATGGGTTGATTTCAAATAAGT
CHS	TGACTATGGTAACATGTCCAGTGC	GAATAACACACCCCAATCCAAAC
CHI	CATCCACACTTCATACTCACAGCTC	TCCTAATGGTGCTTCCATTACTCTT
F3H	ACTTTAGGACTCAAGCGCCACAC	GATCAGCATTCTTGAACCTCCCA
F3'H	TCCATGCTCGGTTTGACGCTTTCC	GGCGTTTTTGGGGATGTGGTAGC
FLS	GCACAAAACAGGTGTACACAAA	TCACCCCAATCCATTTTTAATAG
GT	ACACCTCCAACATGACAGATGATT	AAAAGACGAGATGAGGCTTGAGAT

3.5.) Expression analysis of flavonoid (rutin) biosynthesis genes through qRT-PCR

To study gene expression, equal sample quantities were verified by measuring the amount of RNA with spectrophotometer. The cDNAs were then separated by electrophoresis, stained with ethidium bromide to further verify the equal concentrations. Semi quantitative PCR was performed using following conditions: 94°C for 3 min; 28-35 cycles of 94°C for 30 s, annealing temperature (55-61°C) for 30 s and 72°C for 30 s; and 1 cycle of 72°C for 7 min. *26S rRNA* was used as internal control for expression studies. Gels were scanned with a gel documentation system Alpha Imager EP (Alpha Innotech Corp., USA) and integrated density values (IDV) were calculated with software Alpha Imager EP to determine changes in gene expression.

Gene transcripts showing variation in expression levels at different growth stages were validated again through real-time qRT-PCR. Real-time PCR reactions were performed in duplicate on a CFX 96 system (Bio-Rad Laboratories; Hercules,CA) with the iScript one step RT-PCR Kit (Bio-rad). The PCR protocol was as follows: denaturation for 5 min at 94°C, followed by 40 cycles each of denaturation for 20 s at 94°C, annealing for 30s at 55-61°C, followed by one elongation step for 20 s at 72°C. The significant differences between treatments

were statistically evaluated by standard deviation and heat map was made for folds expression of genes in *F. tataricum* in comparison to *F. esculentum*.

3.6.) Differential transcript profiling through cDNA-AFLP

Rutin content analysis at different growth stages (seed germination to seed maturation; Figure 3.5) showed maximum variation in rutin from flowers to seed maturing stages with relatively higher rutin content in *F. tataricum* compared to *F.esculentum* (Gupta et al., 2011). cDNA-AFLP analysis was performed to identify additional genes (regulators, modifiers, transporters, etc) contributing to higher rutin content in *F. tataricum*.

Double-stranded cDNA was synthesized according to a standard double-stranded cDNA synthesis protocol (Sambrook and Russell, 2001), using DNA polymerase I (*Escherichia coli*) and T4 DNA ligase (NEB, England). About 500 ng of double stranded cDNA was subjected to standard AFLP template production according to Vos et al. (1995). The cDNA was digested with restriction enzymes *Mse*I and *Pst*I (New England Biolabs Inc., Beverly, MA). The digested products were ligated to adapters with sequences as follows: *Mse*I adapter, 5'-GACGATGAGTCCTGAG-3', 3'-TACTCAGGACTCAT-5'; *Pst*I adaptors 5'-CTCGTAGGACTGCGTACATGCA-3', 3'-TGTACGCAGTCTAC-5'. The adapter-ligated DNA served as a template for preamplification, equal amounts of preamplified products were amplified with primers having selective nucleotides at the 3' end (Table 3.4) in a total volume of 20µl. The first selective amplification cycle consisted of 30 s at 94°C, 30 s at 65°C, and 60 s at 72°C. The annealing temperature was lowered by 0.7°C per cycle during the next 12 cycles, followed by 23 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 60 s. To each PCR product was added 7.5 µl of formamide dye and 7 µl of samples were loaded onto a pre-warmed 6% polyacrylamide gel using 1X Tris–borate–EDTA (TBE) buffer and run for 2.5 h at 65 W. The

gels were silver stained using the silver sequence kit (Promega cat. #Q4132, Madison, WI), following the manufacturer's instructions.

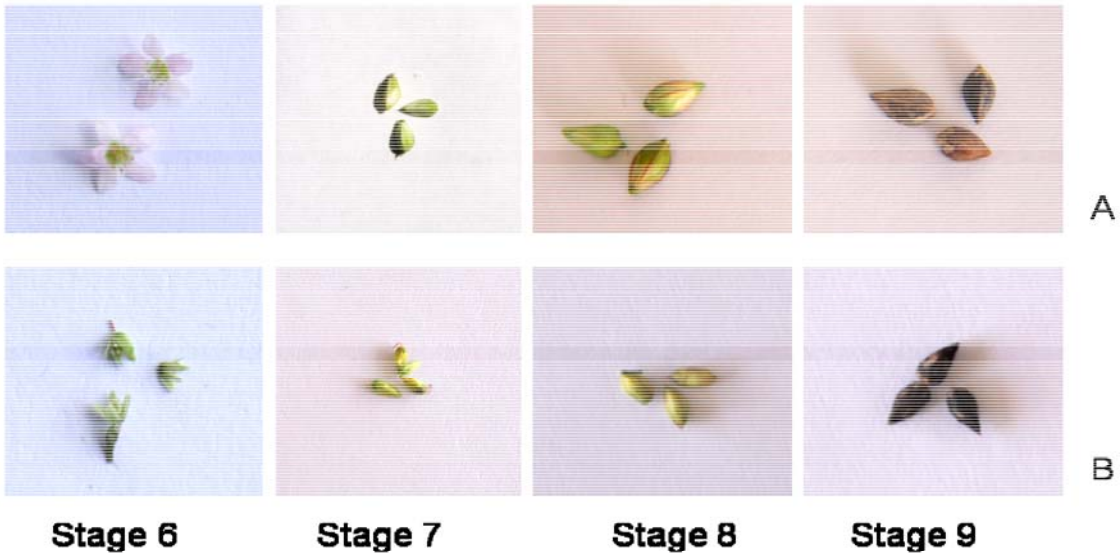

Figure 3.5 Flowers to seed maturing stages of *Fagopyrum* spp. (A) *F. esculentum* (B) *F. tataricum*. Where, Inflorescence (S6); Immature seeds (S7; 12 DAP); Maturing seeds (S8; 22 DAF); Mature Seeds (S9)

Table3.4. Primers used for cDNA-AFLP analysis

Pre-selective primers	**5′-Sequence-3′**
Pst I (00)	GAC TGC GTA CAT GCA G
*Mse*I (00)	GAC GAT GAG TCC TGA GTA A
Selective primers	**5′-Sequence-3′**
Pst I (T)	GAC TGC GTA CAT GCA GT
Pst I (C)	GAC TGC GTA CAT GCA GC
Pst I (A)	GAC TGC GTA CAT GCA GA
Pst I (G)	GAC TGC GTA CAT GCA GG
Pst I (CT)	GAC TGC GTA CAT GCA GCT
Pst I (GA)	GAC TGC GTA CAT GCA GGA
Pst I (GG)	GAC TGC GTA CAT GCA GGG
Pst I (CA)	GAC TGC GTA CAT GCA GCA
Pst I (AT)	GAC TGC GTA CAT GCA GAT
Pst I (CAA)	GAC TGC GTA CAT GCA GCA A
Pst I (CTA)	GAC TGC GTA CAT GCA GCT A
*Mse*I (C)	GAC GAT GAG TCC TGA GTA AC
*Mse*I (G)	GAC GAT GAG TCC TGA GTA AG
*Mse*I (A)	GAC GAT GAG TCC TGA GTA AA
*Mse*I (T)	GAC GAT GAG TCC TGA GTA AT
*Mse*I (CA)	GAC GAT GAG TCC TGA GTA ACA
*Mse*I (GA)	GAC GAT GAG TCC TGA GTA AGA
*Mse*I (TT)	GAC GAT GAG TCC TGA GTA ATT
*Mse*I (AC)	GAC GAT GAG TCC TGA GTA AAC
*Mse*I (AT)	GAC GAT GAG TCC TGA GTA AAT
*Mse*I (AG)	GAC GAT GAG TCC TGA GTA AAG
*Mse*I (TA)	GAC GAT GAG TCC TGA GTA ATA
*Mse*I (CAA)	GAC GAT GAG TCC TGA GTA ACA A

3.6.1) Isolation and re-amplification of transcript derived fragments (TDFs)

The polymorphic transcript derived fragments (TDFs) based on presence, absence or differential expression (fragment intensity) were eluted from the gel in 50µl of sterile double distilled water, incubated at 95°C for 15 min and then hydrated overnight at 4°C. Aliquot of 2µl was used for re-amplification in a total volume of 25 µl, using the same set of corresponding selective primers and the same PCR conditions as used in selective amplification.

3.6.2) Cloning, sequencing and analysis of transcript derived fragments (TDFs)

The amplified TDFs were cloned into plasmid pGEM-T easy® vector (Promega Corp., Madison, WI) following the manufacturer's protocol and then sequenced. The sequences of the TDF were analyzed for their homology against the publicly available non-redundant genes/ESTs/Transcripts in the database (http://www.ncbi.nlm.nih.gov/BLAST, http://www.arabidopsis.org/Blast) using BLASTN and BLASTX algorithms. Sequences where also blasted against the genomes of *Arabidopsis* using FASTA tool (http://www.arabidopsis.org/cgi-bin/fasta/nph-TAIRfasta.pl) developed by TAIR (Rhee et al., 2003), to know the function of transcripts in various metabolic pathways. The TDFs were further classified on the basis of presence/absence and differences in the intensities of fragments.

Real-time RT-PCR analysis

Specific primer pairs were designed for selected TDFs and tested by qRT-PCR (Table 3.5). Primers specific for buckwheat 26S *rRNA* and Histone H3 were used as internal control for the normalization of expression. Real-time PCR reactions were performed as explained above. Whisker plots were drawn for determining folds expression of TDFs in *F. tataricum* in comparison to *F. esculentum* by using MINITAB-14.

Table 3.5 Primer sequences used for qRT-PCR analysis of selected TDFs

Gene Name	Forward Primer Sequence (5'-3')	Reverse Primer Sequence (5'3')
JQ003863	GGGCCTCATTCTTCAACAGG	CCATAGCGAAGTAGTACCGTGAG
JN982731	CACCCCTTTATAAAAGACTTCTTCC	AATCAAGCATCAAAGCTATCAACTC
JN982742	ACAAACAGGTACCATAAAGCCCTAC	GGGCACTATACCTGAGTAACACCTA
JN982718	CTACTGGACTCTCGCCATCTAGG	CTTAGTAGCCAGAGGAAAAGAAAGC
JN982730	CCCACATTACATAACACTCACATCT	GAGACTAGGCCATACACCTTGTTC
JN982732	GTTGATGATAGATGGTAGCGTTTTT	GGAGAGTCGAGTTATTTTGTGAGAG
JN982735	CGTAAGATATCGGCACTCTTTACTC	AAGTAAGAACACCAATGATGAGTCC
JN982734	CACCACGACCAGTATAATGTTGTTA	GAACAAGAAGAGTAAGGAGCACACT
JN982723	AGTCTACTAGCAGCTGAGTCCATGT	GCCTCACCAACTAGCTAATCAGA

Chapter-IV

RESULTS

The results obtained in the present investigation are explained under the following headings:

4.1) Identification of high rutin content accessions of *Fagopyrum tataricum*

4.2) AFLP fingerprinting of *F. tataricum* accessions displaying rutin content variation

4.3) Estimation of rutin content in different growth stages of *Fagopyrum* species

4.4) Cloning and sequencing of structural genes involved in rutin biosynthesis in *Fagopyrum* species through comparative genomics

4.5) Expression analysis of flavonoid (rutin) biosynthesis genes at different growth stages vis-à-vis rutin content variation in *Fagopyrum* species

4.6.) Uncovering additional genes (regulators, transporters, etc.) controlling high rutin content in *F. tataricum* through cDNA-AFLP

4.1) Identification of high rutin content accessions of *Fagopyrum species*

A wide range of rutin content variation was observed among 195 accessions of *F. tataricum*. Most of the accessions (81%) contained seed rutin content in the range of 10-16 µg/mg of rutin, whereas 14% accessions showed considerably higher rutin content (17 µg/mg to 30 µg/mg), few (5%) accessions contained low rutin content (≤10 µg/mg) (Figure 4.1 & Table 4.1). *F.tataricum* accession IC-14889 showed highest rutin content (29.83µg/mg D.W.) followed by IC-42421 (26.86 µg/mg D.W.) and IC-14253 (25.90 µg/mg D.W.). The accessions IC-329457, IC-258233 and IC-329456 containing high rutin contents of 24.03 µg/mg, 18.89 µg/mg, 18.21 µg/mg, respectively belong to rice tartary buckwheat, which is a type of tartary buckwheat

having non-adhering hull. Accessions IC-49676 and IC-310046 contained the lowest rutin contents of 6.85 µg/mg and 6.92 µg/mg, respectively. The high rutin content accessions (Table 4.2) were planted at the experimental farm of NBPGR, Phagli Shimla (H.P.) for validation of rutin content status during the second year (2008-09). From 2 years data (2007-2009), the accession IC-14889 showed highest rutin content (30.21µg/mg) followed by IC-42421 (24.91 µg/mg) and IC-329457 (24.30 µg/mg).

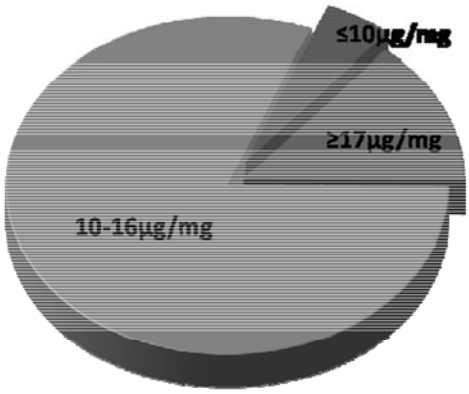

Figure 4.1 Distribution of *F.tataricum* accessions for rutin content

Table 4.1 Rutin and quercetin content variation in mature seeds of different accessions of *F.tataricum*

S.No.	ACCESSION NO.		RUTIN (µg/mg)	QUERCETIN (µg/mg)
1	IC	310045	18.08	0.16
2	IC	310047	12.93	0.13
3	IC	329401	15.13	0.17
4	IC	329456	18.21	0.16
5	IC	341592	12.57	0.19
6	IC	341635	15.19	0.12
7	IC	341651	10.57	0.05
8	IC	341674	15.40	0.12
9	IC	341675	12.22	0.11
10	EC	99948	13.08	0.11
11	IC	540856	13.36	0.08

12	IC	547384	11.43	0.10
13	IC	547388	13.03	0.14
14	IC	547390	12.05	0.08
15	IC	13140	15.76	0.13
16	IC	13143	12.84	0.10
17	IC	14890	14.42	0.13
18	EC	12537	11.76	0.20
19	IC	16535	10.49	0.12
20	IC	16551	14.87	0.12
21	IC	16556	11.93	0.07
22	IC	16579	16.15	0.13
23	IC	17370	13.29	0.12
24	EC	16580	14.82	0.05
25	IC	24299	12.74	0.08
26	IC	26590	12.08	0.11
27	IC	26591	9.76	0.10
28	IC	37277	11.66	0.19
29	IC	37280	10.31	0.09
30	IC	37294	13.33	0.10
31	EC	18282	18.42	0.21
32	IC	46160	11.06	0.13
33	IC	47458	11.89	0.10
34	IC	49160	12.05	0.11
35	IC	49661	12.26	0.12
36	IC	49669	12.94	0.07
37	IC	49675	13.18	0.08
38	EC	18300	13.16	0.15
39	IC	49679	10.20	0.05
40	IC	49681	11.39	0.11
41	IC	49870	10.62	0.02
42	IC	107153	10.74	0.09
43	IC	107208	14.81	0.14
44	IC	107291	13.07	0.07
45	EC	18629	12.55	0.14
46	IC	107293	13.32	0.08
47	IC	107575	13.61	0.11
48	IC	107608	12.00	0.08
49	IC	107616	11.14	0.10
50	IC	107619	10.65	0.08
51	IC	107627	13.29	0.12
52	EC	18740	13.84	0.16
53	IC	107959	10.05	0.07
54	IC	107961	13.92	0.10
55	IC	107968	14.77	0.14

56	IC	107969	12.87	0.13
57	IC	107970	13.42	0.13
58	IC	107983	12.75	0.11
59	IC	107985	11.90	0.12
60	IC	107987	13.54	0.18
61	IC	107989	11.36	0.10
62	IC	107995	11.57	0.09
63	EC	97262	13.43	0.17
64	IC	108500	9.99	0.11
65	IC	108512	11.43	0.11
66	IC	109307	11.81	0.07
67	IC	109725	14.77	0.02
68	IC	274438	11.12	0.11
69	EC	99945	7.79	0.07
70	IC	13412	11.37	0.23
71	IC	313136	23.23	0.33
72	IC	341659	12.66	0.30
73	IC	341661	16.09	0.33
74	IC	13143	14.28	0.25
75	IC	34195	13.55	0.26
76	IC	109309	15.21	0.20
77	IC	274435	11.00	0.15
78	IC	274440	11.59	0.18
79	IC	109316	12.97	0.15
80	IC	329200	13.43	0.22
81	IC	280394	12.92	0.18
82	IC	310095	13.82	0.20
83	IC	310044	13.27	0.32
84	IC	274428	14.31	0.19
85	IC	13140	13.99	0.19
86	IC	14253	25.90	0.30
87	IC	341631	13.53	0.28
88	IC	313125	17.40	0.33
89	IC	13410	15.40	0.27
90	IC	15393	12.41	0.24
91	IC	13411	11.72	0.29
92	IC	329495	12.65	0.26
93	IC	274443	12.94	0.21
94	IC	107974	11.55	0.49
95	IC	107610	10.14	0.43
96	IC	107986	16.52	1.15
97	EC	18282	16.23	0.95
98	IC	107960	10.78	0.57
99	IC	24297	12.06	0.56

100	IC	37291	15.03	0.59
101	IC	107962	20.90	0.90
102	EC	37289	16.23	1.08
103	IC	107583	8.27	0.40
104	IC	108498	15.89	0.98
105	EC	161415	14.21	1.13
106	IC	37282	10.52	0.43
107	IC	22426	13.24	0.75
108	IC	107564	14.53	0.76
109	EC	131602	15.29	1.02
110	IC	258233	18.89	0.57
111	EC	12537	16.91	0.96
112	IC	107967	9.75	0.55
113	EC	99945	14.86	1.03
114	EC	321898	13.48	0.92
115	IC	107984	13.10	0.78
116	IC	108499	16.40	1.22
117	IC	18757	16.08	0.81
118	IC	108503	14.22	0.88
119	IC	108496	15.59	1.32
120	IC	107981	16.03	0.61
121	IC	49676	6.85	0.41
122	IC	18664	9.45	0.02
123	IC	34088	13.21	0.27
124	IC	341577	11.46	0.21
125	IC	341593	12.55	0.25
126	IC	341594	9.62	0.17
127	IC	310046	6.92	0.10
128	IC	37307	13.48	0.24
129	IC	37302	15.50	0.30
130	IC	37310	15.19	0.31
131	IC	42413	16.32	0.26
132	IC	42418	14.31	0.22
133	IC	42421	26.86	0.23
134	IC	42422	15.79	0.23
135	IC	42423	13.41	0.26
136	IC	42424	14.13	0.22
137	IC	42428	16.65	0.28
138	IC	24296	15.89	0.26
139	IC	24298	14.07	0.22
140	IC	24300	10.36	0.16
141	IC	24302	17.20	0.24
142	IC	25794	12.55	0.24
143	IC	25993	14.60	0.24

144	IC	25999	14.23	0.25
145	IC	26552	10.78	0.17
146	IC	26584	13.99	0.20
147	IC	13144	15.23	0.16
148	IC	14889	29.83	0.26
149	IC	341660	16.50	0.21
150	IC	313119	15.76	0.16
151	IC	26756	17.66	0.20
152	IC	26598	11.79	0.20
153	IC	26597	14.17	0.12
154	IC	26596	14.53	0.15
155	IC	26594	12.52	0.17
156	IC	26593	15.65	0.09
157	IC	26592	12.62	0.11
158	IC	26589	15.45	0.12
159	IC	26588	17.28	0.09
160	IC	26587	14.36	0.09
161	IC	37265	17.04	0.17
162	IC	37278	14.16	0.10
163	IC	37279	15.85	0.13
164	IC	37286	14.65	0.11
165	IC	37288	13.51	0.12
166	IC	37290	14.42	0.12
167	IC	37295	16.82	0.14
168	IC	37297	15.74	0.15
169	IC	37299	12.03	0.11
170	IC	37301	14.11	0.11
171	IC	341591	19.209	0.046
172	IC	108514	19.106	0.106
173	IC	108516	17.175	0.112
174	IC	108518	15.315	0.067
175	IC	109237	17.209	0.168
176	IC	109211	15.177	0.123
177	IC	108904	18.676	0.081
178	IC	108510	15.526	0.109
179	IC	108519	16.562	0.121
180	IC	16558	16.996	0.172
181	IC	341581	20.019	0.180
182	IC	14494	19.203	0.184
183	IC	274331	23.030	0.177
184	IC	49672	19.094	0.175
185	IC	49671	19.892	0.237
186	IC	49667	22.220	0.237
187	IC	49666	19.873	0.196

188	IC	49664	19.064	0.229
189	IC	49663	17.131	0.244
190	IC	49662	19.966	0.176
191	IC	49660	18.452	0.182
192	IC	47929	18.010	0.146
193	IC	42431	19.960	0.206
194	IC	42429	20.429	0.169
195	IC	329457	24.03	0.121

Table4.2 Rutin content in mature seeds of high rutin content accessions of *F. tataricum*

Accession Number	Rutin content (µg/mg) in Yr I	Rutin content (µg/mg) in Yr II	Mean rutin content for 2 Yrs (µg/mg)
IC-49667	22.22±0.12	21.97±0.10	22.09
IC-274331	23.04±0.18	20.37±0.53	21.71
EC-18282	18.42±0.50	21.96±0.12	20.19
IC-14253	25.90±0.10	22.23±0.20	24.06
IC-313136	23.23±0.20	19.45±0.17	21.34
IC-14889	29.83±0.37	30.58±0.44	30.21
IC-310045	18.08±0.12	19.06±0.13	18.57
IC-107962	20.70±0.14	21.51±0.39	21.11
IC-42421	26.86±0.40	22.96±0.13	24.91
IC-26756	17.66±0.33	22.99±0.11	20.33
IC-258233	18.89±0.31	19.32±0.27	19.11
IC-329456	18.21±0.25	17.89±0.03	18.10
IC-329457	24.03±0.12	24.57±0.07	24.30

*One way ANOVA revealed significant differences between different types of accessions ($p<0.0001$), between different years ($p<0.0001$), and genotypes x year interactions ($p<0.0001$).

4.2) AFLP fingerprinting of *Fagopyrum tataricum* accessions displaying rutin content variation

AFLP fingerprinting of high (Table 4.2) and low rutin content accessions, IC-49676 (6.85 µg/mg), IC-310046 (6.92 µg/mg), EC-99945 (7.79 µg/mg), IC-107583 (8.27 µg/mg) and IC-18664 (9.45 µg/mg) was carried out by using 19 AFLP primer combinations of *Eco*RI/*Mse*I. In total 907 clearly visible fragments were detected out of which 136 were polymorphic with an average of 7.15 polymorphic fragments per primer combination. The number of fragments

generated for each primer combination ranged from 22 for *Eco*RI-TAG/*Mse*I-AAG to 72 for *Eco*RI-A/*Mse*I-A with an average of 47.73 fragments per primer combination. The number of polymorphic bands ranged from 2 for *Eco*RI-TAG/*Mse*I-AAG and *Eco*RI-TGG/*Mse*I-TCC to 18 for *Eco*RI-A/*Mse*I-G. The extent of polymorphism was highest (29.5%) for *Eco*RI-A/*Mse*I-G followed by 29.19% for *Eco*RI-G/*Mse*I-C and 21.21% for *Eco*RI-AA/*Mse*I-CAT primer combinations. Lowest polymorphisms of 5.45% and 6.66% were observed in primer combinations *Eco*RI-C/*Mse*I-CT and *Eco*RI-AA/*Mse*I-TCC, respectively (Table 4.3).

The highest PIC value (0.288) was obtained for *Eco*RI-AA/*Mse*I-AC followed by 0.286 for *Eco*RI-G/*Mse*I-C and 0.283 for *Eco*RI-A/*Mse*I-G. The lowest PIC value per primer combination was 0.045 and 0.063 for *Eco*RI-C/*Mse*I-CT and *Eco*RI-GA/*Mse*I-AC, respectively.

Population genetic structure

Hierarchical cluster analysis (UPGMA) showed that high and low rutin content accessions were grouped separately into two clusters (Figure 4.2). Cluster I further divided into 3 clusters, cluster-Ia contained all low rutin content accessions (IC-49676, IC-310046, IC-170583, IC-18664 and EC-99945); cluster-Ib contained high rutin accessions of tartary buckwheat (IC-26756, IC-107962) and cluster-Ic contained high rutin accessions of rice-tartary buckwheat (IC-329457, IC-329456 and IC-258233). Cluster II contained all the high rutin content accessions (IC-14253, EC-18282, IC-274331, IC-49667, IC-14889, IC-313136, IC-310045 and IC-42421). To further test this population structure, a model-based clustering method was implemented in the program STRUCTURE. Without prior information about the populations and under an admixed model, STRUCTURE calculated that the estimate of the likelihood of the data (LnP(D)) was greatest when K= 2. For K.2, LnP(D) increased slightly (Figure 4.3a), i.e., DK reached its maximum at K= 2 (Figure 4.3b), suggesting that all populations fell into one of the two clusters.

The red cluster covered all low rutin content accessions along with few high rutin accessions, and the other high rutin accessions were grouped in the green cluster (Figure 4.3c). This result is identical to the splitting in the NJ tree. Furthermore, UPGMA Cluster I and II are identical to the red and green clusters, respectively, indicating that the grouping of all 18 populations is well supported. Analysis of Molecular Variance (AMOVA) based on the two genetic clusters indicated that majority of genetic variation (72%) occurred within populations, while the variation between populations was 28% (Table 4.4).

Population genetic diversity

The genetic diversity (H) computed among different groups of populations was recorded in between 0.171 for low rutin accessions to 0.274 for high rutin accessions. The Shanon's information index (I) obtained was recorded in the range of 0.250 for low rutin accessions to 0.408 for high rutin accessions. Based on all accessions, the observed number of alleles was 1.84 and the effective number of alleles was found to be 1.49 per locus. Similarly, the total genetic diversity (Ht) among the genotypes was 0.29 and within genotypes (Hs) was 0.25. Shannon's information index was 0.44 and estimated gene flow was found to be 1.34 among the 18 *F. tataricum* accessions (Tables 4.5 & 4.6). The results of this analysis reveal that the genetic differentiation among population is significant and that gene flow is restricted.

Table4.3. Total number of bands, polymorphic bands, percent polymorphism, polymorphic information content (PIC) obtained with 19 AFLP primer combinations on 18 accessions of *F.tataricum*

Primer Pair	Total bands (No.)	Polymorphic bands (No.)	Percent polymorphism (%)	PIC
*Eco*RI(G)/*Mse*I(C)	62	15	24.19	0.286
*Eco*RI(A)/*Mse*I(G)	61	18	29.5	0.283
*Eco*RI(A)/*Mse*I(C)	71	12	16.90	0.135
*Eco*RI(A)/*Mse*I(A)	72	17	23.61	0.238
*Eco*RI(A)/*Mse*I(G)	68	6	8.82	0.075
*Eco*RI(AA)/*Mse*I(AC)	52	8	15.38	0.288
*Eco*RI(GA)/*Mse*I(AC)	42	3	7.14	0.063
*Eco*RI(AA)/*Mse*I(TTC)	63	6	9.52	0.128
*Eco*RI(AA)/*Mse*I(TGA)	42	5	11.90	0.168
*Eco*RI(AA)/*Mse*I(TCC)	45	3	6.66	0.068
*Eco*RI(AA)/*Mse*I(CAT)	33	7	21.21	0.225
*Eco*RI(AC)/*Mse*I(GA)	32	5	15.62	0.207
*Eco*RI(AC)/*Mse*I(AA)	36	4	11.11	0.161
*Eco*RI(TAG)/*Mse*I(AAG)	22	2	9.09	0.131
*Eco*RI(TGG)/*Mse*I(TCC)	25	2	7.14	0.083
*Eco*RI(TCC)/*Mse*I(TGA)	32	6	18.75	0.231
*Eco*RI(TA)/*Mse*I(AAG)	43	9	20.93	0.238
*Eco*RI(A)/*Mse*I(GA)	51	5	9.80	0.112
*Eco*RI(C)/*Mse*I(CT)	55	3	5.45	0.045
Total	907	136	272.72	3.165
Mean	47.73	7.15	14.35	0.167

Table 4.4 Summary of analysis of molecular variance (AMOVA) based on high vs. low accessions of *F. tataricum*

Source of variance	d.f	S.S.D.	Variance Component	Percentage	P-value
Among populations	2	52.933	3.485	28	< 0.001
Within populations	15	136.067	9.071	72	< 0.001
Total	**17**	**191.000**	**12.557**	**100**	

Levels of significance, d.f.: degree of freedom; S.S.D.: sum of square deviation; P-value: probability of null distribution

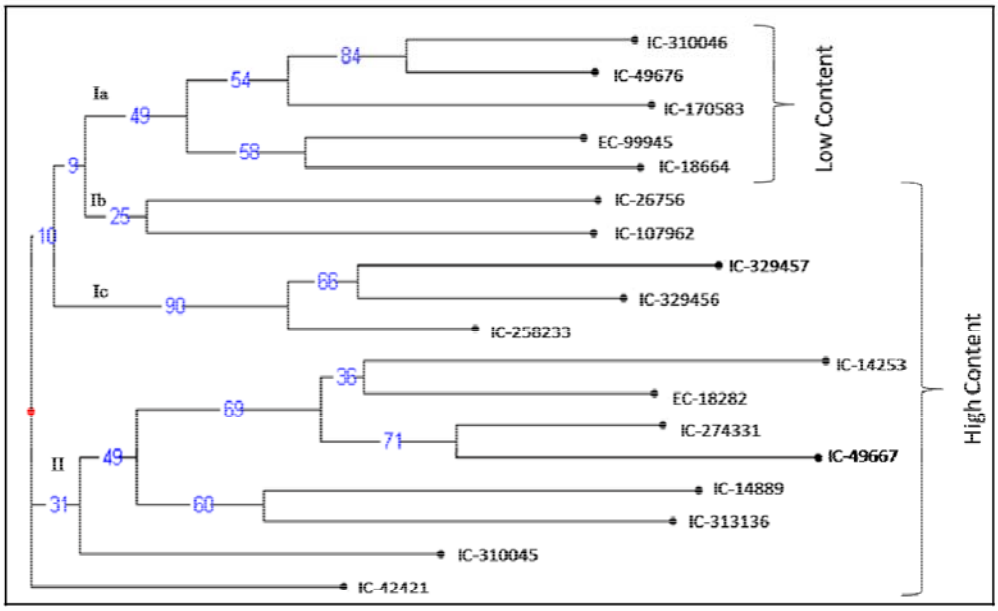

Figure 4.2 Dendrogram of genetic relationship among high and low rutin content accessions of *F.tataricum* based on AFLP analysis

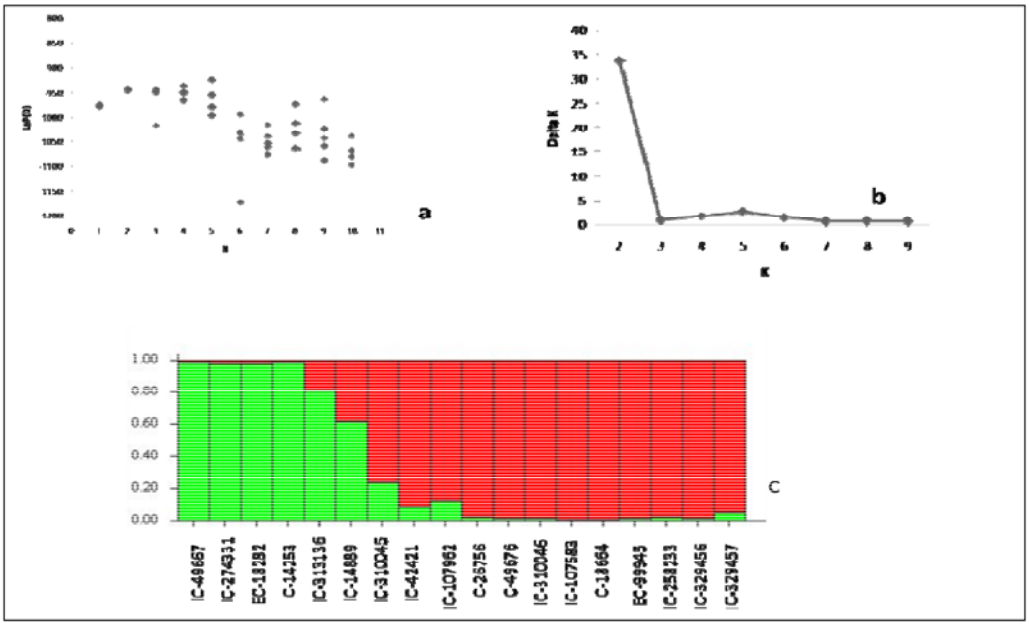

Figure 4.3 STRUCTURE analyses of *F. tataricum* populations. Based on AFLP data (a: the relationship between K and LnP(D); b: the relationship between K and DK; c: the grouping when K = 2).

Table 4.5 Summary of genetic variation statistics among the accessions of *F. tataricum*

Sample	Sample size	Na	Ne	H	I	Ht	NPL	PPL
Low rutin	5	1.425 (0.498)	1.299 (0.381)	0.171 (0.207)	0.250 (0.299)	0.171 (0.043)	31	42.47
High rutin	13	1.753 (0.434)	1.470 (0.370)	0.274 (0.189)	0.408 (0.264)	0.274 (0.036)	55	75.34

*Na, Observed number of alleles; Ne, Effective number of alleles; H, Nei's gene diversity; I, Shannon's Information index; Ht, Heterozygosity; NPL, Number of polymorphic loci; PPL, Percentage of polymorphic loci

Table 4.6 Overall genetic variability across all the 18 accessions of *F. tataricum* based on AFLP marker

Sample size	Na	Ne	H	I	Ht	Hs	NPL	PPL	Gst	Nm
18	1.849 (0.360)	1.484 (0.326)	0.291 (0.167)	0.439 (0.230)	0.291 (0.028)	0.245 (0.025)	62	84.93	0.157	1.342

*Gene flow (Nm) = 0.25(1 - Gst)/Gst

4.3) Estimation of rutin content in different growth stages of selected genotypes of *Fagopyrum* species

High rutin content accessions; IC-14889 (30.21µg/mg) of tartary buckwheat, IC-329457 (24.30µg/mg) of rice-tartary buckwheat and IC-540858 (0.72µg/mg) of common buckwheat were selected for molecular dissection of flavonoid biosynthesis machinery in *Fagopyrum* species. Rutin content estimation in nine growth stages (S1 to S9; Figure 3.2) of *Fagopyrum* species from seed germination to mature seed formation was done. The stages S1 and S2 of tartary buckwheat IC-14889 (3.5 folds, 4.6 folds, respectively) and rice tartary buckwheat IC-329457 (4.1 folds & 4.6 folds, respectively) showed comparatively higher rutin content in comparison to common buckwheat IC-540858. The rutin content increased exponentially from

stages S3 to S6 of IC-540858, whereas it showed fluctuating patterns of biosynthesis and accumulation in stages S3 to S5 in the order of S5>S3>S4 of IC-14889 and IC-329457 (Figure 4.4). The inflorescence stage (S6) had the highest rutin content in IC-329457 (15.75 µg/mg f.w.) compared to IC-540858 (10.42 µg/mg f.w.) and IC-14889 (10.26 µg/mg f.w.) (Figure 4.4). The rutin content decreased during post-flowering stages, S7 & S8 with a sharp decrease in common buckwheat compared to tartary and rice tartary buckwheat. The rutin content was ~17 folds higher at stage S7 in IC-14889 and ~31 folds higher in IC-329457 compared to IC-540858, which then decreased in stage S8 to ~4.7 folds and ~8 folds, respectively. The increase in rutin content was highest at fully developed seeds (S9) of *F. tataricum* with ~55 folds in IC-14889 and ~43 folds in IC-329457 compared to IC-540858 of *F. esculentum* (Figure 4.4; Table 4.7).

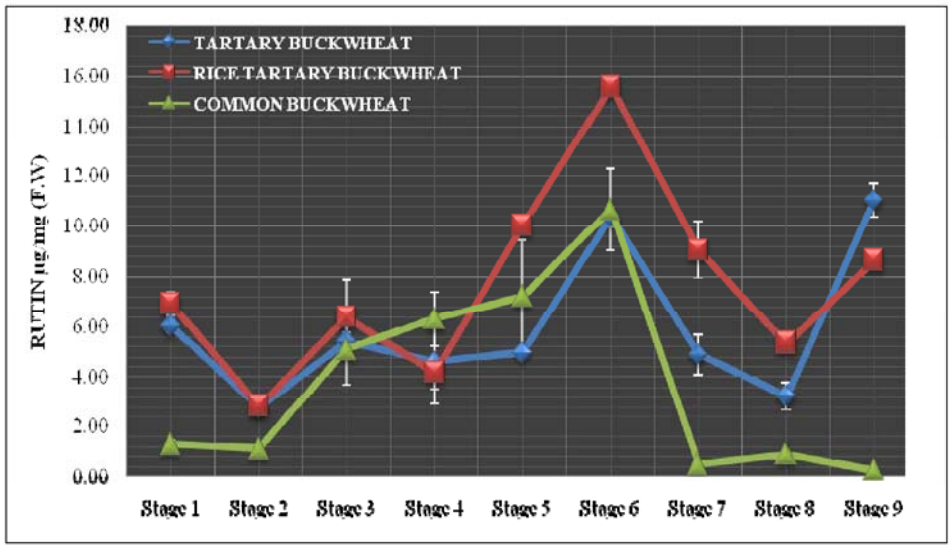

Figure 4.4 Rutin content variation (fresh weight basis) in different growth stages of *Fagopyrum sp.* Accessions: Tartary Buckwheat (IC-14889), Rice Tartary Buckwheat (IC-329457), and Common Buckwheat (IC-5408858)

Table 4.7 Variation (x) in rutin content in different growth stages of Tartary (*F. tataricum*) and Rice tartary buckwheat (*F. tataricum*) with respect to common buckwheat (*F. esculentum*)

Stage	Growth stage	Tissue/ Sample taken	Rutin content (x times) compared to common buckwheat	
			Tartary buckwheat	Rice- tartary buckwheat
Stage 1	Emergence	Whole seedling	3.5	4.1
Stage 2	First pair of leaves	Whole seedling	4.6	4.6
Stage 3	True leaves formed	True Leaf	1.14	1.3
Stage 4	Leaf growth	Mature Leaf	-1.4*	-1.5*
Stage 5	Flowering & mature leaves	Mature Leaf	-1.5*	1.4
Stage 6	Peak flowering	Inflorescence	1	1.5
Stage 7	Seed formation started	Immature Seeds	17	31
Stage 8	Seeds are in the dough stage	Immature Seeds	4.7	8
Stage 9	Filled seeds are brown	Mature brown Seeds	55	43

*Rutin content higher in common buckwheat

4.4.) Cloning and sequencing of structural genes involved in rutin biosynthesis in buckwheat through comparative genomics

The flavonoid biosynthetic pathway structural genes were isolated from *F. tataricum* through comparative genomics with other dicot plant species. By using PCR with primers designed at conserved sequence region of genes, the fragments of PAL, 4CL, C4H, CHI, F3H, FLS and F3'H were isolated (Figure 4.5). The fragment size of PAL was 1.14 kb and amplicons for C4H, 4CL, F3H, F3'H and FLS genes ranged from 180-350 bp. The sequence of gene fragments for PAL, C4H, FLS F3H, showed 70-95% identity with the corresponding genes from other species, whereas gene fragments for F3'H, 4CL and CHI showed 50-70% of identity (Table 4.8). More than one sequence was found from isolated cDNA fragments of C4H, F3H and F3'H, however, the fragments showing maximum identity with the genes from other species were selected for designing of primer pairs for gene expression analysis on *Fagopyrum* species.

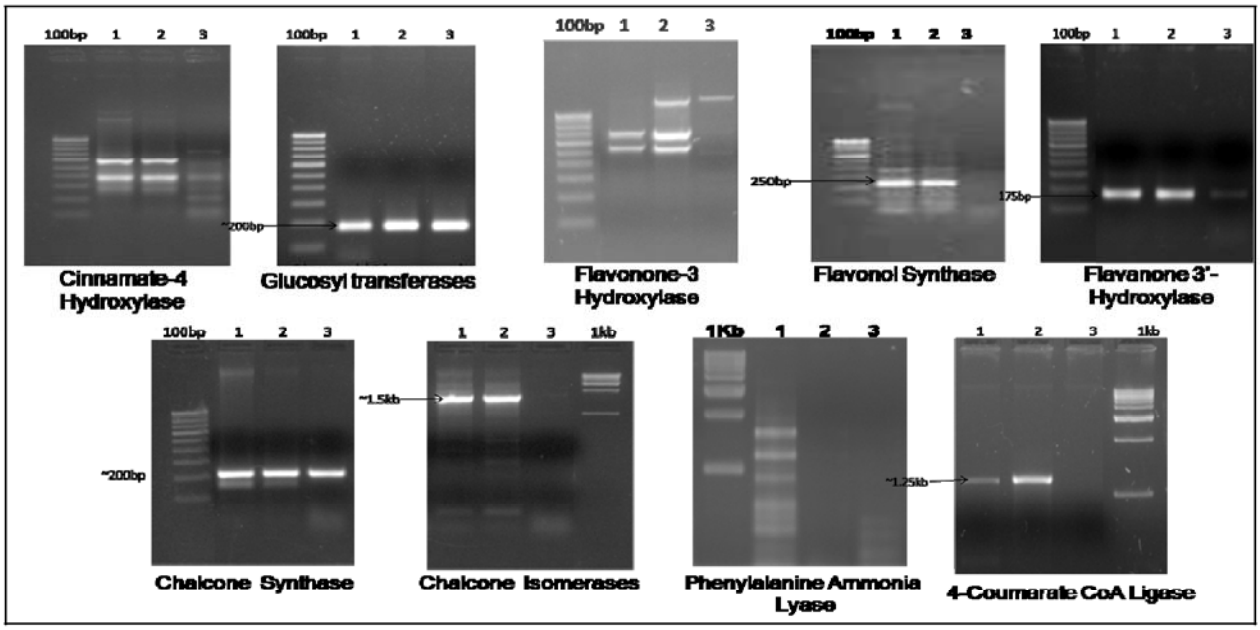

Figure 4.5 Amplification of rutin biosynthetic pathway genes on genomic DNA (gDNA) of *Fagopyrum* species

Table 4.8 Comparison of the deduced amino acid sequences of flavonoid pathway structural genes from *F.tataricum* with other plant species

Gene / Fragment size	GenBank Accession Number	Plant Species	Identity (%)	Ref ID (Protein)
PAL (1144bp)	HQ828145	*Daccus carota*	90	BAG31930
		Nicotiana tabacum	91	ACJ66297
		Beta vulgaris	90	CAH17686
		Brassica napus	91	ABC69917
		Rhodiola sachalinensis	90	AAW78932
C4H (343bp)	HQ828144	*Brassica rapa*	79	BA117481
		Salvia miltiorrhiza	80	ABC75596
		Helianthus tuberosus	77	CAA78982
		Arabidopsis thaliana	76	AAC99993
4CL (421bp)	HQ844966	*Glycine max*	70	AAL98709
		Populous trichocarpa	58	XP002324477
CHI (332bp)	HQ844964	*Aquilegia Formosa*	50	ABA86593
		Rhododendron x pulchrum	52	BAF96948
F3H (189bp)	HQ202162	*Camellia japonica*	87	BAI66079
		Gossypium hirsutum	85	ADC96713
		Parsea Americana	84	ACA65272

		Dimocarpus longan *Vitis vinifera*	84 82	ABO48521 ABM67589
F3'H (259bp)	HQ833212	*Vitis vinifera* *Vitis amurensis*	50 52	XP00284165 ACN38268
FLS (233bp)	HQ833211	*Rosa hybrid* *Fragaria x ananassa* *Ricinus communis* *Pyrus communis*	80 70 75 75	BAC66468 ABH07784 XP002513774 ABB70188

4.5.) Expression analysis of flavonoid (rutin) biosynthesis genes at different growth stages vis-à-vis rutin content variation in *Fagopyrum* species

In order to understand the contribution of a particular flavonoid (rutin) biosynthesis gene(s) towards rutin content variation, the quantitative RT-PCR analysis was done at different growth stages of both the *Fagopyrum* species *i.e.* rice tartary (IC-329457) and common buckwheat (IC-540858). Tissue samples from six growth stages, S2, S5, S6, S7, S8 & S9, which had shown significant variations in rutin content, were selected for expression analysis (Table 4.7, Figure 4.4). No significant variation was observed in the expression levels of C4H, 4CL, GT, F3'H and F3H genes (Table 4.9), whereas four genes, 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 (Figure 4.6). The PAL transcript abundance was highest in stage S6 (flowers) of both the *Fagopyrum* species which then decreased during the subsequent seed developmental stages, S7, S8 and S9 (Figure 4.6). In comparison to *F.esculentum*, the expression of PAL was 7.69 higher in S2 (seedlings) of *F. tataricum* (Figure 4.7). The PAL expression increased exponentially in seed maturation stages S7, S8 and S9 with 3.14, 5.66 and 8.93 folds higher expression in *F. tataricum* respectively. The expression of CHS gene was highest in stage S6 followed by S9 of both the *Fagopyrum* species with relatively higher transcript abundance in *F. tataricum* compared to *F. esculentum* (2.13 and 3.19 folds

higher, respectively). Similarly, the CHI expression level was higher in S6 and S7 with an increase of 4.45 and 5.66 folds higher expression in S6 and S7 respectively of *F. tataricum* compared to *F. esculentum*. Likewise, the expression level of FLS gene was observed to be relatively higher in different growth stages of *F. tataricum* compared to *F. esculentum* with the maximum increase in transcript level (4.61 folds) during stage S6. Whereas, the transcript abundance of FLS gene was 6.1 folds higher in stage S2 of *F. esculentum* in comparison to *F. tataricum*.

Table 4.9 Folds expression of flavonoid (rutin) biosynthesis genes in *F. tataricum* over *F. esculentum*

Gene Name	Stages					
	S2	S5	S6	S7	S8	S9
PAL	7.69	1.71	1.57	3.14	5.66	8.93
4CL	-1.13*	1.02	1.09	1.09	-1.02*	-1.02*
C4H	1.02	1.02	-1.03*	-	-1.01*	-1.01*
CHS	1.49	-1.05*	2.13	1.86	2.19	3.19
CHI	1.59	1.29	4.45	5.66	1.08	2.98
F3H	1.01	-1.01*	-	1.02	1.02	1.01
F3'H	-1.01*	-1.01*	-1.01*	-	-1.01*	1.03
FLS	-6.1*	1.41	4.62	1.21	1.3	1.21
3GT	1.01	1.01	1.01	-	-1.01*	1.01

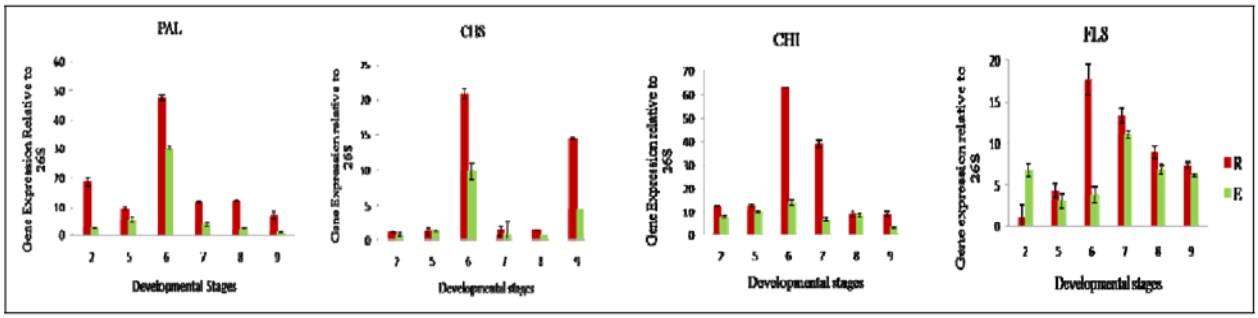

Figure 4.6 Expression level of rutin biosynthetic pathway genes through qRT PCR at different growth stages (S2, S5, S6, S7, S8 & S9) of buckwheat. The expression level of each gene is relative to that of the constitutively expressed 26S gene. R– Rice Tartary Buckwheat (IC-329457) and E – Common Buckwheat (IC540858)

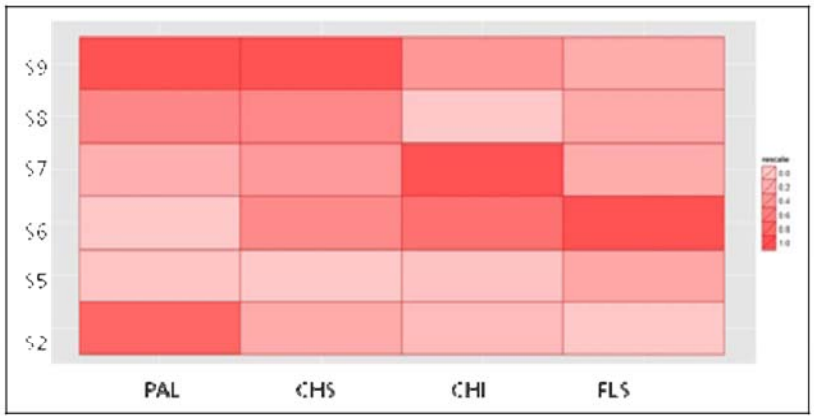

Figure 4.7 Heat Map depicting folds expression of rutin biosynthesis genes in different growth stages of *F. tataricum* over *F. esculentum*

4.6.) Uncovering additional genes (regulators, transporters, etc.) controlling high rutin content in *F. tataricum* through cDNA-AFLP

4.6.1) Identification and analysis of differentially expressed transcripts (TDFs)

cDNA-AFLP analysis on RNA samples from flower to mature seed stages of rice-tartary and common buckwheat with 32 primer pair combinations resulted in the identification of 42 clear and unambiguous fragments (TDFs) with each primer combination. The TDFs ranged in size from 150-750 bp representing a total of 2,584 TDFs for flower to mature seed stages of both the *Fagopyrum* species.

A total of 167 differential TDFs based on presence/ absence or differences in level of intensity were eluted from the gels, re-amplified and sequenced (Figure 4.8). The DNA sequence of each TDF was assigned a putative biological function by checking against the Gene Bank database (BLASTN/BLASTX) as well as the TAIR database. The TDFs represented genes controlling different biological processes such as general and secondary metabolism (33%), regulation (18%), signal transduction (14%), transportation (13%), cellular organization (10%),

transposable elements (7%), photosynthesis (4%) and defense & response to stimuli (1%) (Table 4.10, Figure 4.9).

The TDFs encoding for enzymes such as alanine glyoxlate, methionine sulfoxide, fatty acid desaturase (FAD), KAS III, sucrose 6 phosphatase, ubiquitin protein ligases etc. were implicated in the biosynthesis of proteins & amino acids (12%), fatty acids (7%) and carbohydrates (6%). Similarly, 8% of TDFs corresponded to key enzymes involved in secondary metabolism, including the flavonoid and anthocyanin biosynthesis. The TDFs (10 %) involved in cellular function included genes coding for pectin acyltransferases, prolein rich extensins, glycine rich proteins, and arabinogalactan, etc. The TDFs corresponding to genes encoding transporters (13%) included ABC transporters, auxin hydrogen symporter, sugar transporters, and zinc and potassium transporters. Genes involved in signal transduction (14%) and gene regulation (18%), including Zn finger binding proteins, Leucine rich repeats calmodulin binding protein, protein kinases and transcription factors belonging to MYB and WRKY classes were also detected.

The TDFs representing differentially expressed genes were classified into different categories on the basis of their presence/absence (qualitative variation) or differences in level of expression (quantitative variation) between both the *Fagopyrum* species so as to infer whether TDFs belonging to a particular functional class are preferentially expressed in a particular *Fagopyrum* species (Figure 4.10A & B). The TDFs encoding for carbohydrate metabolism and signal transduction were relatively higher in number in *F. esculentum* whereas, TDFs encoding for secondary metabolism, amino acid & protein metabolism, energy and photosynthesis were more in *F. tataricum* (Figure 4.10 A). Most of the TDFs encoding for transporters, transcriptional regulation, secondary metabolism, photosynthesis & energy, carbohydrate,

protein and amino acid metabolism showed relatively higher expression pattern in *F. tataricum* compared to *F. esculentum* (Figure 4.10 B). Whereas, only TDFs for cellular metabolism showed relatively higher expression in *F. esculentum* compared to *F. tataricum.*

The TDFs representing genes involved in transport of metabolites (ABC and sugar transporters, auxin hydrogen symporter), regulation of biosynthesis (MYB TF, Zn finger protein), metabolism of metabolites (ubiquitin protein ligases, extensin protein), signal transduction (calmodulin binding protein) and energy transfer (ATP CF0 subunit) were chosen for quantitative RT-PCR analysis in different seed maturing stages of both the *Fagopyrum* species.

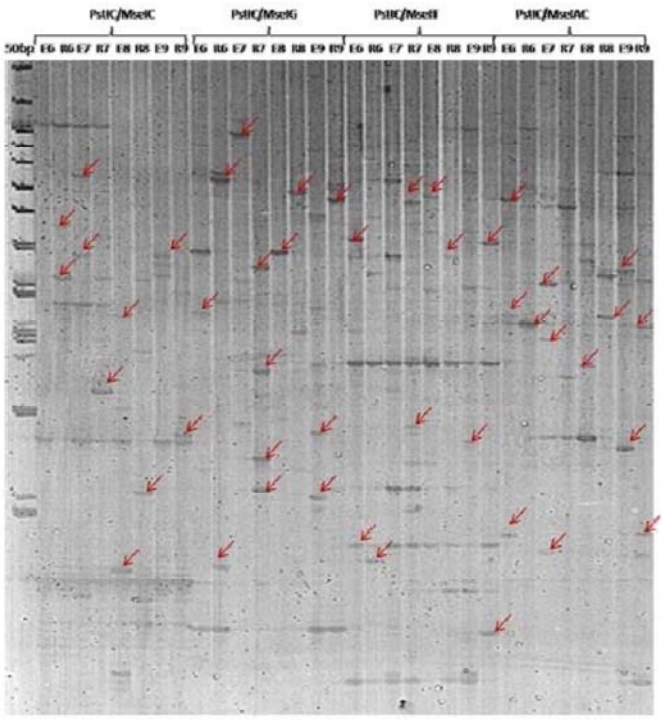

Figure 4.8 Expression pattern of *F.tataricum* (R) vs. *F.esculentum* (E) transcripts in seed maturing stages displayed by cDNA-AFLP. An example showing selective amplification with four different primer combinations, where 6, 7, 8 and 9 are flower to seed maturing stages.

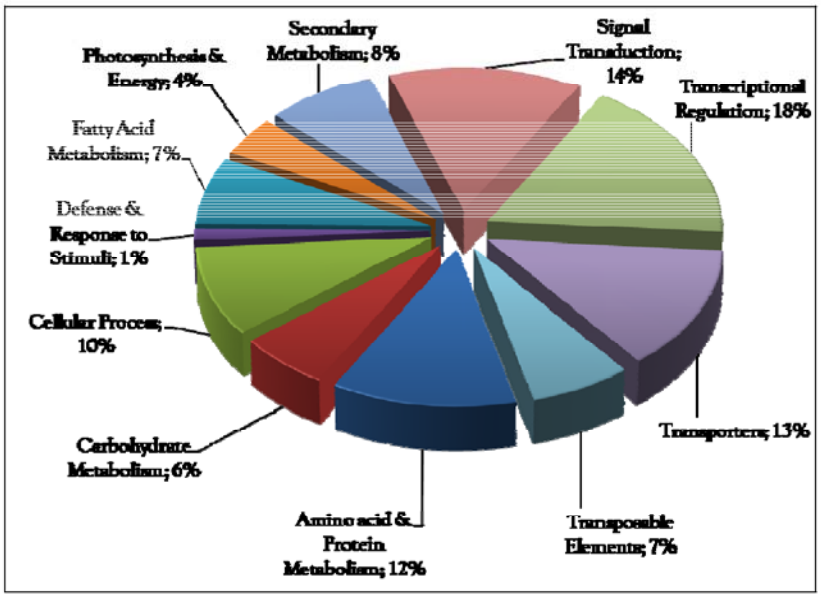

Figure 4.9 Functional classification of transcript fragments

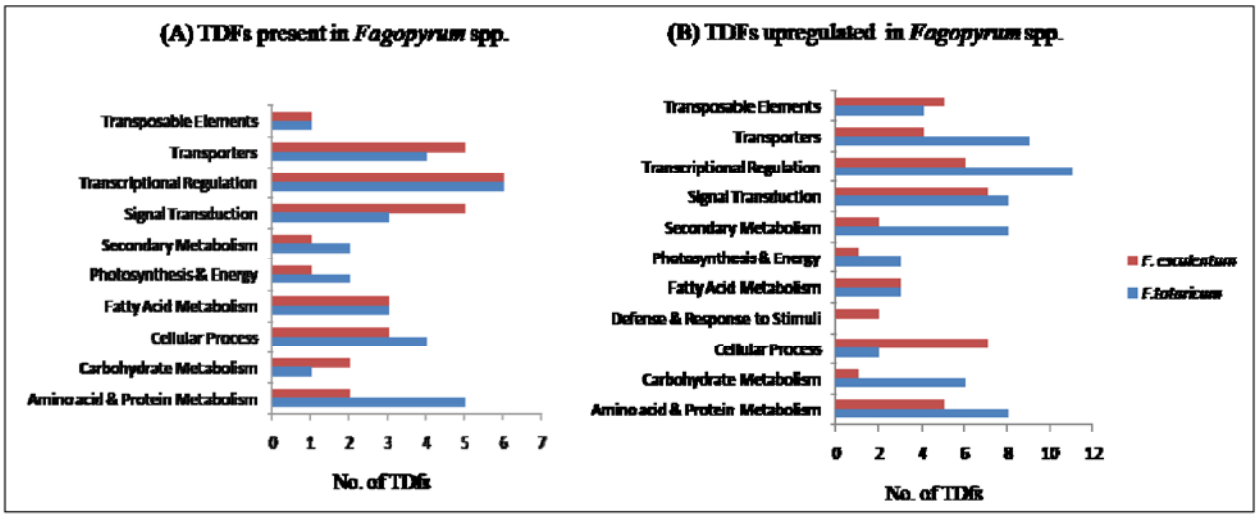

Figure 4.10 Functional classification of differentially expressed TDFs in *Fagopyrum* species

4.6.2) Transcript quantification of selected TDFs in flower to seed maturation stages of Fagopyrum spp.

Four TDFs encoding Ubiquitin protein ligase (JN982742), ABC transporter (JN982732), sugar transporter (JN982735) and MYB118 (JN982734) showed significantly higher expression in flowers (S6) of rice-tartary compared to common buckwheat with 2.86, 4.71, 7.36 and 11.42 folds expression, respectively (Figure 4.11A). Relatively higher expression was observed for TDFs encoding for ABC transporter, sugar transporter, Ub protein ligase and Zn finger binding protein (JN982723) in immature seed stage (S7) of rice-tartary buckwheat with 95.38, 49.25, 18.92, 17.29 folds, respectively over common buckwheat (Figure 4.11B). On the other hand, 5 TDFs encoding Zn finger binding protein, ATP CF0 subunit (JN982718), calmodulin binding protein, extensin (JQ003863) and auxin efflux (JN982731) showed relatively higher expression in the immature seeds (Stage S8) of common buckwheat compared to rice-tartary buckwheat with 22.22, 11.94, 4.04, 3.89, and 1.64 folds higher expression respectively. Two TDFs encoding ABC transporter (2.38 folds) and MYB118 (1.61 folds) continued relatively higher expression in S8 of rice-tartary in comparison to common buckwheat (Figure 4.11C). In mature seeds (S9), the expression of TDFs for Zn finger binding protein (12.54 folds), ABC transporter (6.78 folds), Ub protein ligase (2.54 folds), calmodulin binding protein (2.32 folds), and sugar transporter (2.07 folds) was found to be higher in *F. esculentum* compared to *F. tataricum* (rice-tartary buckwheat). Whereas, extensin and ATP CF0 subunit showed relatively higher expression in *F. tataricum* over *F. esculentum* in mature seeds (S9) with 5.42 and 2.09 folds respectively (Figure 4.11D). No transcript was detected for MYB 118 and auxin efflux carrier protein in mature seeds of rice-tartary buckwheat. Therefore, the transcripts of ubiquitin protein ligases, ABC transporter

and sugar transporter showed relatively higher expression in three stages of seed development, including flowers and immature seeds of rice-tartary compared to common buckwheat.

The expression pattern of 9 TDFs was also checked in vegetative tissues (leaf, stem and roots) of both the *Fagopyrum* species so as to infer their contribution in biosynthesis, transport or regulation of metabolites because the content of rutin were minimal in these tissues in both *Fagopyrum* species. Out of 9, 4 TDFs, ABC transporter, MYB 118, ubiquitin protein ligase and Zn finger binding protein also showed relatively higher expression in vegetative tissue of rice-tartary buckwheat (Figure 4.11E). The TDF encoding ABC transporter showed 22.5, 10.37 and 3.2 folds higher expression in roots, leaves and stems of rice-tartary compared to common buckwheat, respectively.

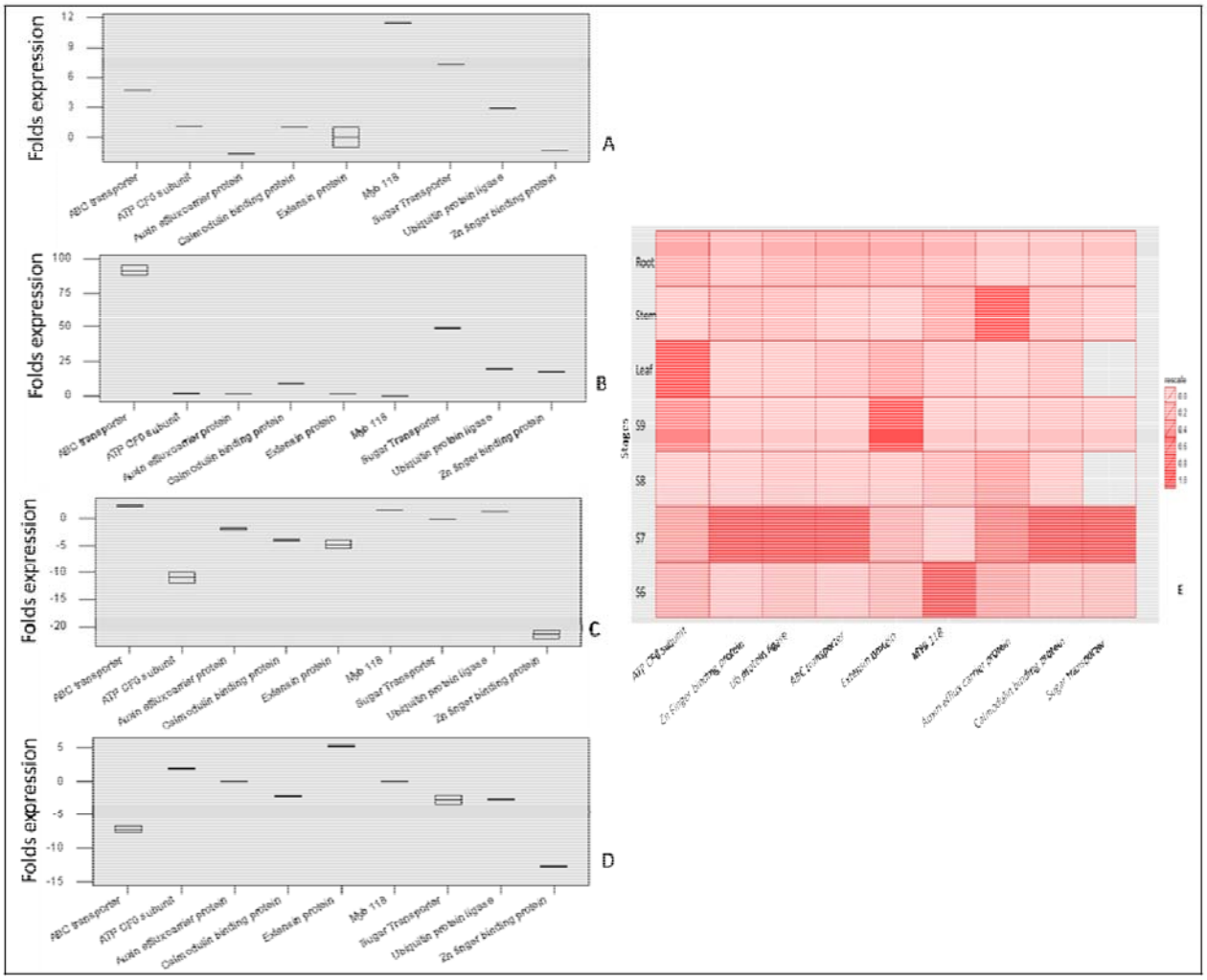

Figure 4.11 Expression of TDFs in different tissues of *Fagopyrum* species. Fold expression in rice-tartary buckwheat was calculated in comparison to their expression in different tissues of common buckwheat. A: Flowers (S6); B: Immature seeds (S7); C: Immature seeds (S8); D: Mature seeds (S9); E: Heat map of fold expression of TDFs expressed in seed maturing stages and vegetative tissues (leaf, stem and root) of *F. tataricum* in comparison to *F. esculentum*

Table 4.10 Sequence homology of transcript derived fragments (TDFs) based on BLAST X and TAIR FASTA analysis

Sequence Name	Accession Number	Homology to gene (Genebank ID)	Organism	E-value Score	Function	Metabolic Pathway
Secondary Metabolism						
N_M-G_F5	JN982719	Dihydroflavonol 4 reductase (ACG42172.1)	*Zea mays*	2e-05	Anthocyanin Biosynthesis	Anthocyanin biosynthesis
16a_MG	JN982729	Cinnamoyl CoA reductase (AAY86360.1)	*Acacia auriculiformis X Acacia mangium*	1e-12	Key enzyme of monolignoids	Lignin biosynthesis
Dc	JN982733	Chalcone synthase (ACH70135.1)	*Fagopyrum tataricum*	4e-27	Key enzyme of flavonoid biosynthesis	Flavonoid biosynthesis
Q	JN982746	Cinnamoyl CoA reductase family (XP_002526624.1)	*Ricinus communis*	0.014	Key enzyme of monolignoids	Lignin biosynthesis
4A_T	JQ417192	NAD(P)-Binding Rossamann Fold (AT1G09500)	*Arabidosis thaliana*	0	Cinnamoyl CoA dehydrogenase activity	Phenylpropanoid biosynthesis, lignin biosynthesis
15a	JN982728	Cytochrome P450 family (AT5G52320)	*Arabidosis thaliana*	1.8e-06	Catalyze the oxidation of various intermediates	Limonene and pinene degradation, Stilbenoid, diarylheptanoid and gingerol biosynthesis, Biosynthesis of secondary metabolites
27b	JQ417191	UDP Glucosyl Transferases (AT5G03490)	*Arabidosis thaliana*	1.3e-189	Transfer of glucose to flavonol	Flavone and flavonol biosynthesis (ath00944), Glycosylation regulates transportation, solubility
Transporters						
2B_P-C	JN982721	Zinc Transporter (XP_002517414.1)	*Ricinus communis*	5e-04	Transportation of Zn ions	Cation transmembrane transporter activity, transportation of Zn (micronutrient) from soil to roots and then to various plant

						parts
A2	JN982731	Auxin efflux carrier protein / Auxin:Hydrogen symporter (NP_197014.1)	*Arabidopsis lyrata*	0.007	Transportation of auxins	Regulation of auxin homestasis within plant cells
Db	JN982732	ATP binding cassette transporter putative (XP_002516304.1)	*Ricinus communis*	9e-05	Transportation of flavonoids	Transportation of flavonoids from cytosol to vacuoles
Dh	JN982735	Sugar transporter protein (NP_001191180.1)	*Zeamays*	3.2	Carbohydrate transmembrane transport, proton transport	Transportation of sugars to cells for growth and development, and for synthesis of storage compounds
K1	JN982744	Putative high affinity potassium transporter (BAD31835)	*Oryza sativa*	1e-06	Transportation of K+ ions	K^+ uptake from soils into roots and from roots to different cells within plants
6_M-T	JQ003853	MATE efflux family protein (AT1G61890)	*Arabidopsis thaliana*	6.6e-06	Transmembrane transport of metabolites	Vacuolar sequestration of flavonoids, efflux of toxic compounds etc
9_GA088	JQ003854	Transmembrane amino acid Lys/His transporter (AT1G47670.1)	*Arabidopsis thaliana*	6.5e-05	Transportation of amino acids Lys/His	Transportation of amino acids during seed germination and grain filling
24a	JQ003855	Glucose-6-phosphate antiporter/ triose-phosphate transmembrane	*Arabidopsis thaliana*	1.4e-50	Transportation of glucose-6-phosphate	Glucose 6-P transportation in plastids for starch biosynthesis and pentose phosphate pathway

		transporter (AT5G46110.3)				
E2	JQ003856	Sec23/Sec24 protein transporter (AT4G32640)	*Arabidopsis thaliana*	0	ER to golgi vesicle mediated protein transport	Protein processing in endoplasmic reticulum (ath04141)
Photosynthesis and energy						
Ec	JN982738	NADH dehydrogense subunit F (ABP88019.1)	*Scoparia dulcis*	5.5	Energy, ATP synthase copled electron transport	Oxidative phosphorylation
F_MG_049	JN982718	Putative ATP CF0 subunit (CAO02552.1)	*Vigna unguiculata, Fagopyrum esculentum*	3e-03	Oxidative phosphorylation	Translocation of protons across the membrane
Da PAT		Phosphoenolpyruvate carboxylase (AT3G61530.1)	*Arabidopsis thaliana*	3.6e-39	Catalyses beta oxidation of PEP	Involved in C4 photosynthesis in mesophyll cells
Fatty Acid Biosynthesis						
C1	JQ003857	GroES-like zinc-binding dehydrogenase (AT1G32780.1)	*Arabidopsis thaliana*	1.2e-06	Alcohal dehydrogenase activity	Fatty acid metabolism (ath00071)
Dh_PAT	JQ417190	Phosphatidic acid phosphatase (AT5G03080.1)	*Arabidopsis thaliana*	1.5e-62	Lipid synthesis by dephosphlylating phosphatidic acid	N-Glycan biosynthesis (ath00510)
11B	JQ417186	ACC Biotin containing subunit (AAC49114.1)	*Arabidopsis thaliana*	1e-07	Carboxylation of Acetyl CoA to Malonyl CoA	Fatty acid biosynthesis.
Ea	JQ003858	3-ketoacyl-CoA synthase (AT3G52160.1)	*Arabidopsis thaliana*	8.4e-05	Condensation of a malonyl-ACP with an acyl acceptor	Fatty acid elongation (ath00062)
J-1	JN982743	Lipoxygenase (AAF15296.2)	*Phaseolus vulgaris*	5e-05	catalyzes the addition of molecular oxygen	In defense against pathogens, fruit ripening, nitrogen storage,

					to polyunsaturated fatty acids to produce an unsaturated fatty acid hydroperoxide	nodule development etc.
Fd	JN982740	Phospholipase C6 (NP_190430.2)	*Arabiodopsis thaliana*	1.2	Cellular regulation, lipid metabolism, membrane remodeling	Hydrolyze phospholipids for the release of secondary messenger that participate in different signal transduction pathway
30	JQ003861	GDSL-like Lipase/Acylhydrolase (AT1G20130.2)	*Arabiodopsis thaliana*	0	Hydrolysis of ester bonds	Lipid metabolism
Cellular process						
11b1	JN982725	Nodulin (NP_194482.1)	*Arabidopsis thaliana*	1e-05	nodulin like protein L2	Reproduction of plants, symbiosis
23a	JQ003859	Actin binding (AT5G07740)	*Arabiodopsis thaliana*	3e-201	Rearrangement of the actin cytoskeleton	Regulates cell growth and morphology
Dk	JQ003862	Pectin acetylesterase family protein (AT3G62060.1)	*Arabiodopsis thaliana*	1e-141	Cell wall modification	Hydrolyze acetyl esters in pectin
E1	JQ003863	Proline-rich extensin-like family protein (AT5G19810.1)	*Arabiodopsis thaliana*	8.9e-05	Major proteins in plant cell wall	Important role in various biological processes such as embryo development, root hair growth, seed coat development, defense etc.
1-2	JN982747	ribosomal protein L2 (ATCG01310.1)	*Arabidopsis thaliana*	3.3e-06	Structural constituent of ribosome	Ribosome (ath03010)
4A_A	JQ003865	75K gamma secalin (ADP95516.1)	*Triticum aestivum*	0.045	Nutrient reservoir activity	Storage protein
Protein metabolism/ Amino acid biosynthesis						
Dm_MAG	JQ003866	Eukaryotic aspartyl protease (AT4G16563.1)	*Arabidopsis thaliana*	0.049	Proteolysis	Cleavage of specific dipeptide bonds

11b_085	JQ417188	Chaperone DnaJ-domain superfamily (AT1G62970.1)	*Arabidopsis thaliana*	2.3e-05	Heat shock protein	Helps in control of protein disaggregation
2B_M-C	JN982720	Cysteine protease (AAP41486.1)	*Anthurium andraeanum*	2e-05	Proteolytic activity	Involved in protein maturation, degradation, and protein rebuilt in response to different external stimuli
22a	JN982742	Ubiquitin protein ligase (EEE30515)	*Ricinus communis*	2e-04	Protein degradation	Protein degradation
11_MCA	JN982724	Pyridoxal-5'-phosphate-dependent Threonine synthase (AT1G72810.1)	*Arabidopsis thaliana*	1e-84	Threonine biosynthesis process	Glycine, serine and threonine metabolism Vitamin B6 metabolism (ath00750)
AN14	JQ003869	Alanine:glyoxylate aminotransferase (AT4G39660.1)	*Arabidopsis thaliana*	1.9e-43	Glyoxylate transaminase activity	Alanine, aspartate and glutamate metabolism (ath00250), Glycine, serine and threonine metabolism (ath00260)
10-a1	JQ003870	Peptidylprolyl Isomerase (AAF75383.1)	*Arabidopsis thaliana*	3e-04	Isomerisation of dipeptide bonds	Protein scaffolding
Transcriptional regulation						
10b_MC	JN982723	Zinc finger family protein (XP 0028927381.1)	*Arabidopsis lyrata*	0.003	Transcriptional regulation	Inactivation of transcription, flower and seed development, seed differentiation, stress tolerance
Di	JN982736	Histone acetyl transferase (NP_001105145.1)	*Zeamays*	7e-04	Transcriptional regulation	Gene regulation, acylation of histone and non-histone proteins like transcription factors, nuclear receptors to facilitate gene expression
4A_T	JN982722	GA MYB Like 2	*Solanum*	4e-04	Important role in	Mediate GA signaling

		(ABM53274.1)	*lycopersicum*		pollens and anther development in response to gibberellic acid	
Dg_PAT	JN982734	MYB 118 (XP_002877072.1)	*Arabidopsis lyrata*	3e-04	Transcriptional regulation	Embryogenesis and seed development & maturation in *Arabidopsis*
10a2	JQ003871	MYB112, AtMYB112 | myb domain protein (AT1G48000.1)	*Arabidopsis thaliana*	6.3e-129	Transcription factors	Transcriptional regulation
18b	JQ003852	FH5; formin homolog protein 5 (AAS93430.1)	*Arabidopsis thaliana*	4.1	Transcriptional regulation	Mediate ubiqutination, signal transduction, regulation of cell cycle, transcriptional regulation
Carbohydrate Metabolism						
K3	JN982745	Starch branching enzyme 3 (ABU41261)	*Oryza sativa*	1e-04	Starch biosynthesis by introduction of branch points	Starch metabolism
17a	JQ417183	UDP-D-apiose/UDP-D-xylose synthase (AT2G27860.1)	*Arabidopsis thaliana*	7e-05	Sugar biosynthesis	Amino sugar and nucleotide sugar metabolism (ath00520), hemicelluloses synthesis
36_PCT	JQ417189	Sucrose-6F-phosphatase (AT3G52340.1)	*Arabidopsis thaliana*	5.9e-55	Sucrose biosynthesis	Final step in sucrose biosynthesis
18x1	JQ003860	GDP Mannose 4,6 dehydrogense	*Arabiodopsis thaliana*	1.8e-25	metabolism	Cell wall carbohydrate synthesis, protein glycosylation
Signal Transduction						
36	JN982730	Calmodulin dependent protein kinase	*Zeamays*	2.1	Acting as a secondary messengers through signal transduction	Regulate diverse cellular processes by interacting with other proteins

		(AAG01179.1)				
27	JQ003868	GTP binding protein subunit (XP_002519124)	*Ricinus communis*	2e-04	Signal transduction	Control of plant growth, differentiation and development in responses to biotic and abiotic stress
Dq	JN982737	Protein kinases (ABA9926)	*Oryza sativa*	6e-06	Catalyze the reversible transfer of the y-phosphate from ATP to amino acid side chains of proteins.	Regulating cellular division, cell differentiation and morphogenesis
Ed	JN982739	Receptor like protein kinase (ACM89561.1)	*Glycine max*	0.94	Signal Transduction	Control protein activity and cellular signaling
j058	JQ003864	Leucine-rich repeat (LRR) family protein (AT3G05990.1)	*Arabidopsis thaliana*	3e-116	Signal Transduction	Plant LRR proteins involved in such diverse processes as pollen tube growth, root development, Ran GTPase activation, transcription regulation and meristem cell organization
Defense and Response to stimulus						
29	JQ417184	TIR disease resistance protein (NP_001185276)	*Arabidopsis thaliana*	7e-05	Defense response	Defense against pathogens

Chapter-V

DISCUSSION

Buckwheat (*Fagopyrum* spp.), is the only nutraceutical food crop known as the richest source of rutin, an important flavonoid of medicinal value. Dissection of flavonoids biosynthetic machinery in a nutraceutical food crop with high rutin content offers avenues towards identification of novel genes with their potential utilization in genetic improvement or metabolic engineering. The present study was taken up for understanding the molecular basis of higher flavonoid (rutin) content in *F. tataricum* in comparison to *F. esculentum* so as to utilize information in genetic improvement. The available *F. tataricum* accessions from North-Western Himalayas were utilized for the identification of high rutin containing accessions. A detailed analysis of results on rutin content estimation, AFLP fingerprinting, expression analysis of flavonoid biosynthesis pathway genes along with differential transcript profiling of *Fagopyrum* species has been described with possible explanations in the following sections:

Rutin content profiling of *F. tataricum* accessions

Flavonoids are ubiquitous plant secondary metabolites that are implicated in diverse metabolic functions, including their role in stress protection. Rutin is one of the important flavonoid, which provides protection against reactive oxygen species and has various pharmacological properties (Chauhan et al., 2010; Kuntić et al., 2011). The flavonoids content variation within species was studied in different plants like Barrenwort, Potentilla, Ginger, Spinach, Strawberry and Citrus etc. (Ghafar et al., 2010; Tomczyk et al., 2010; Quan et al., 2010; Steffensen et al., 2011). Rutin

content has also been quantified in various plants *Eucalypus*, Amaranthus, Apple, cherry etc (Kalinova and Dadakova 2009; Attanassova and Bagdassarian 2009).

Rutin being the flavonoid of nutraceutical value in *F. tataricum* (Campbell 1997) necessitates that high rutin content accessions should be identified from naturally occurring genetic resources. Variation in rutin content was reported earlier for 7 genotypes of *F. tataricum* (Yan et al., 2004) based on differences in ecological conditions from where the collections were made. In addition, rutin content variation was detected (13µg/mg to 20µg/mg) among 50 strains of tartary buckwheat on the basis of differences in seed shape and color (Park et al., 2004). In the present study, significant variation for rutin content (6µg/mg to 30µg/mg) was observed among 195 accessions of *F.tataricum* collected from the North-Western Himalayan regions of India grown at same environmental conditions so as to reflect genotypic variation (Figure 4.1; Table 4.1). The variation in rutin content was found to be consistently stable for 2 years, thereby, implying that genetic differences do exist in *F. tataricum* accessions for rutin content variation. Hence, high rutin content accessions identified can be utilized for their cultivation and conservation. The *F. tataricum* and *F. esculentum* accessions with contrasting variation in rutin content can be used not only in genetic improvement but also in cloning useful genes/alleles contributing to high rutin content.

AFLP fingerprinting of high and low rutin content accessions of *F. tataricum*

The correlation between genetic diversity and secondary metabolite content has been studied through various DNA fingerprinting techniques (RAPD, AFLP and ISSR) in different plant species such as Ashwagandha (Dhar et al., 2006), *Podophylum* (Sultan et al., 2010), and *Fructus xanthii* (Han et al., 2008), thereby, demonstrating genetic diversity for secondary metabolites content. Positive correlation between hypericin content and genetic structure of *Hypericum*

strains was demonstrated through AFLP analysis (Tonk et al., 2011). These studies implied that molecular markers can be used to correlate genetic variability with the contents of secondary metabolites. High genetic variability was also found among *F. esculentum* populations based on morphological and physiological characters (Iwata et al., 2005; Cepková et al., 2009). Genetic variability has also been characterized in tartary buckwheat cultivars through AFLP markers (Zhao et al., 2002; Zhang et al., 2007; Hou et al., 2009). Although, none of these studies looked at correlating DNA fingerprinting with any morphological or physiological trait including rutin content in buckwheat.

In our AFLP analysis, 19 primer combinations out of 28 showed clear polymorphisms in 18 *F. tataricum* accessions including high and low rutin content. Out of which three primer combinations, *Eco*RI-A/*Mse*I-G, *Eco*RI-G/*Mse*I-C and *Eco*RI-AA/*Mse*I-CAT were effective in grouping high vs. low rutin content accessions. The average polymorphism per primer combination was 14.35%, which were similar to (15.7%) previous studies in *F.tataricum* (Hou et al., 2009). In population genetics, a value of gene flow (Nm) < 1.0 (less than one migrant per generation into a population) or equivalently, a value of gene differentiation (Gst) > 0.25 is generally regarded as the threshold quantity beyond which significant population differentiation occurs (Slatin 1987). The results of this analysis revealed that the genetic differentiation among population is 0.157 indicating that genetic diversity resides within the accessions. The results of our hierarchical and model-based cluster analyses of AFLP data strongly revealed that the high and low rutin content accessions of *F. tataricum* were clustered separately (Figure 4.2 & 4.3). The AFLP analysis, therefore, successfully grouped high and low rutin accessions of *F.tataricum* into separate clusters. Strong correlation was also reported for secondary metabolite content and RAPD profile among six *Hypericum* species from Serbia (Smelcerovic et al., 2006). The

parallelism between the contents of withanolides and AFLP fingerprinting among *Withania somnifera* populations suggested an appreciable degree of congruence between the two (Dhar et al., 2006). Therefore, AFLP fingerprint profiles specific to high rutin content can assist in quality control for selective cultivation of high rutin accessions and conservation of superior ones.

Estimation of rutin content in different growth stages of *Fagopyrum* species

Estimation of rutin content in different growth stages of *Fagopyrum* species revealed that the biosynthesis and accumulation of rutin content vary during different growth stages of tartary and common buckwheat (Figure 4.4; Table 4.7). Both the accessions (IC-14889, IC-329457) of *F. tataricum* showed more or less similar pattern of rutin biosynthesis and accumulation in different growth stages compared to *F. esculentum* (IC-540858). The rutin content increased exponentially during post flowering stages (S6-S9) of *F. tataricum* compared to *F. esculentum*. The higher amounts of rutin content at seedling stages (S1 and S2) of *F. tataricum* compared to *F. esculentum* can be attributed to the relatively higher amounts of rutin stored in the seeds of *F. tataricum*, which may be required for physiological functions during early seedling growth. The rutin content increased in an exponential fashion from stage S2 to S6 and then decreased sharply during the subsequent stages of seed formation and development in *F. esculentum*. On the other hand 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 patterns of rutin content variation have been variable in different plant species. For example the rutin content has been reported to be same in different growth stages of *Hypericum perforatum* such as 0.87% in roots, 0.84% in leaves, and 0.8% in flowers of (Sofic et al., 2010), whereas variable in different growth stages of American elderberry with 0.67% in leaves, 0.55% in flowers, and 0.018% in green stems. The highest amount of rutin observed in flowering stage of both the *Fagopyrum* species is

analogous to previous reports (Wagenbreth et al., 1996; Park et al., 2004). The amounts of secondary metabolites have been found to be maximum in the flowers followed by a decrease during the later stages of seed development in *Ipomea sp.* (Khatiwora et al., 2010), *Achillea* (Karlová 2006), *Artemesia annua* (Baraldi et al., 2008) and *Vitis vinifera* (Boss et al., 2006). In American cranberry, the concentration of flavonoid compounds was observed to be highest in flower ovaries which then declined during fruit development and then increased during maturation (Vvedenskaya and Vossa 2004). Hence, 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*.

Cloning and expression profiling of flavonoid pathway genes vis-à-vis rutin content variation in different growth stages of *F.tataricum*

Understanding the differential expression of flavonoid pathway genes would help in discerning molecular mechanisms contributing to higher rutin content in *F. tataricum* over *F. esculentum.* In order to study the expression pattern of flavonoid pathway genes in different growth stages of *Fagopyrum* species, genes PAL, C4H, 4CL, CHI, FLS, F3H and F3'H were isolated through comparative genomics from *F. tataricum*. More than one gene fragment was detected for PAL, C4H and F3'H genes in buckwheat which suggested the presence of multiple copies of these genes in buckwheat genome as has been observed in other plant species (Han et al., 2010, Li et al., 2010). In *Arabidopsis*, most of the flavonoid pathway genes occur in single copies (Winkel-Shirley 2001); whereas in legumes, most of the genes occur in multigene families (Mathesius et al., 2001). For multi-copy gene sequences, the critical gene sequence putatively involved in rutin biosynthesis was deciphered through literature survey and targeting particularly those genes

which have been demonstrated to be involved in flavonoid biosynthesis through gene function analyses such as gene silencing or over-expression.

A significant difference in the level of expression between the genes was found through quantitative RT-PCR analysis (Figure 4.6 & 4.7). Out of 9 genes involved in rutin biosynthesis; genes encoding PAL, CHS, CHI and FLS showed differential expression patterns at different growth stages of *Fagopyrum* species. The transcripts of these genes were found to be relatively higher in *F. tataricum* in comparison to *F. esculentum* which is in concurrence with rutin content. The expression of these genes was observed to be highest in the inflorescence stage of both the species, which correlated with the highest rutin content. PAL is a basic cellular enzyme associated with a host of functions and biosynthesis of diverse phenylpropanoids (Chang et al., 2009). The PAL expression increased exponentially in seed maturing stages S7, S8 and S9 with 3.14, 5.66 and 8.97 folds higher expression respectively. PAL catalyzes the flux of primary metabolites into flavonoid biosynthetic pathway, thereby, plays a key role in the biosynthesis of flavonoids (Tao et al., 2011). The PAL activity was also found to be maximum in flowers (Stage S6) of both the species of buckwheat compared to the other growth stages (Jaakola et al., 2002). The higher transcript abundance of PAL in the seeds of *F. tataricum* can be correlated with high rutin content because fully developed seeds (S9) of *F. tataricum* contained 43-55x higher rutin compared to *F. esculentum*. The expression of CHS gene was highest in stage S6 and S9 with relatively higher expression in *F. tataricum*. Both these stages also contain higher amounts of rutin, therefore, a positive correlation was observed between the expression of CHS gene and rutin content in buckwheat. The CHS gene has been found to be a rate limiting step in the flavonoid biosynthesis in different plants as studied through RNAi silencing and over-expression (Bovy et al., 2007; Pitakdantham et al., 2010; Zhou et al., 2011b). The high activity of CHS has

also been reported in the flowers of tree peony (Zhou et al., 2011b). The CHI gene required for the conversion of naringenin chalcone to naringenin is also considered an essential gene for the biosynthesis of flavonoids (Park et al., 2011a; Povero et al., 2011). In the present study, the level of CHI transcript was comparatively higher in S6 and S7 of *F.tataricum* compared to same stages of *F. esculentum*. The expression of CHS and CHI genes was also positively correlated with the flavonoid accumulation in Citrus and Litchi (Bovy et al., 2007; Wang et al., 2010). FLS gene also showed significant variation in its expression at different growth stages of *Fagopyrum* species, which is concurrent with the role of FLS gene in flavonoid biosynthesis pathway (Jiang et al., 2010; Ali et al., 2011). A significant increase of 4.61 folds in the transcript of FLS gene in the flowers of *F. tataricum* compared to *F. esculentum* was observed, though there was ~1.5 folds increase in the rutin content in S6 of *F. tataricum*. In other plant species such as Citrus, *Arabidopsis,* Tomato, etc; FLS gene has been considered to be as rate limiting step in biosynthesis of flavonoids (Bovy et al., 2007). The expression of other flavonoid pathway genes C4H, 4CL, F3H, F3'H and GT didn't show any significant variation in their transcript level in different growth stages of *Fagopyrum* species (Table 4.9). Therefore, the positive correlation of 4 flavonoid pathway genes, PAL, CHS, CHI and FLS with the rutin content variation at different growth stages of *Fagopyrum* species is found to be encouraging. Further analysis and cloning of promoter regions of those genes from *F. tataricum* and *F. esculentum* would provide insights into what determines their differential expression between *Fagopyrum* species.

Differential transcript profiling to capture genes regulating biosynthesis, modification, transport and accumulation of flavonoids in *Fagopyrum* spp.

The flavonoid content in a particular tissue and developmental stage is largely influenced by different classes of regulatory genes, transporters, etc. in addition to structural genes of flavonoid

biosynthetic pathway (Grotewold 2006; Bovy et al., 2007; Hichri et al., 2011). Understanding types and number of genes differentially expressed during seed maturation would help in discerning molecular mechanisms contributing to nutritional superiority and morphological variations in seeds of *F. tataricum* over other *Fagopyrum* species. The fagopyritols, rutin and sucrose contents are found to increase significantly during seed maturation stages of buckwheat (Horbowicz et al., 1998; Obendorf 1998; Gupta et al., 2011). De-novo sequencing of transcripts from flowers of *Fagopyrum* species (*F. tataricum* and *F. esculentum*) had shown that the genes contributing to different biological processes are contributing to variations in the morphology of flowers in *Fagopyrum* species (Logacheva et al., 2011). Molecular dissection of the genetic machinery contributing to nutritional and morphological differences in the seeds of *F. tataricum* over *F. esculentum* was undertaken through cDNA-AFLP since it is an open architecture technology for global transcriptional analysis in non-model species (Bachem et al., 1996; Meyers et al., 2004).

Large numbers of TDFs with differential expression pattern were observed in different seed developmental stages of both the *Fagopyrum* species. TDFs with differential expression patterns belonged to genes involved in primary and secondary metabolism, transportation, signal transduction, gene regulation, photosynthesis & energy, defense and cellular processes in seed developmental stages of the *Fagopyrum* spp (Figure 4.10). Out of 167 differential TDFs, only 22 showed 50-70% identity with the available floral transcriptome of both the *Fagopyrum* species (Logacheva et al., 2011), thereby, suggesting that most of the TDFs identified in the current study are new. The TDFs encoding genes involved in transport, transcription, secondary metabolism, amino acid & protein metabolism, carbohydrate metabolism and photosynthesis were relatively higher in number and expression pattern in *F. tataricum* over *F. esculentum.* The

higher expression in *F. tataricum* TDFs involved in secondary metabolism and transportation such as chalcone synthase, dhihydroflavonol reductase, UDP glucosyl transferases, ABC transporters, MATE efflux carrier proteins, which are known to be involved in biosynthesis, accumulation and transportation of flavonoids, indicate their involvement in significantly higher flavonoid content in this species (Grotewold 2006; Park et al., 2011a; Zhao et al., 2011). Upregulation of TDF encoding for Lys/His transporter in *F. tataricum* was implicated for higher amount of histidine in this species (Kusano et al., 1992). The TDFs involved in amino acid & protein metabolism (like Ub protein ligases, alanine glyoxylate amino trnasferases, cystein proteases), transcriptional regulation (MYB 118, MYB 112, GAMYB, histone acetyl transferases) and signal transduction (calmodulin binding protein, protein kinases, PEP carboxylase) were also found to be up-regulated in *F. tataricum*. Most of these TDFs represent genes with their direct or indirect role in controlling the growth and development of seeds and/or their nutritional composition. On the basis of differential expression pattern of transcripts in *F. tataricum* and *F. esculentum*, the TDFs, representing genes which have been implicated in biosynthesis, modification, regulation and transport of secondary metabolites (Grotewold 2006; Hichri et al., 2011; Zhao et al., 2011) were chosen to investigate their role, through qRT-PCR analysis in higher rutin content or other metabolites in seeds of *F. tataricum* over *F. esculentum* (Figure 4.11).

The flavonoid content increase in buckwheat seedlings has been attributed to the increase in the concentration of sucrose (Li et al., 2011). In addition, sugars also act as developmental signals regulating seed maturation and accumulation of flavonoids in plants such as *Arabidopisis, V. vinifera,* etc. (Wobus and Weber 1999, Solfanelli et al., 2006, Ferri et al., 2011). The exponential increase in the transcript of a sugar transporter (JN982735) from flowers (S6) to

immature seeds (S7) of rice-tartary compared to common buckwheat suggests its contribution to higher content of flavonoids and fagopyritols in the seeds of *F. tataricum*. Slighter increase in the transcript of sugar transporter in the roots of rice-tartary buckwheat and stem of common buckwheat indicate its involvement in the transport of sugars towards other metabolic processes such as cell wall metabolism, plant growth because these vegetative tissues are otherwise very low in flavonoid and fagopyritol contents (Steadman 2000; Zhang et al., 2011). Relatively higher expression of auxin efflux carrier protein (JN982731) in different seed maturation stages (S8 & S9) as well as in leaves and roots of *F. esculentum* suggests its negative role in the biosynthesis of flavonoids, which are present in lower amounts in different growth stages of *F.esculentum* (Gupta et al., 2011). Flavonoids have been implicated as inhibitors of auxin transport in *Arabidopsis* (Brown et al., 2001, Hichri et al., 2011). The ABC and MATE classes of transporters are known to be involved in the transport of flavonoids from cytosol into vacuoles (Grotewold 2006; Buer et al., 2007; Zhao et al., 2011). In our study the expression of ABC transporter (JN982732) was relatively higher in stages S6 (4.7x), S7 (95.4x), and S8 (2.4x) of rice-tartary buckwheat compared to common buckwheat. In addition, the level of expression of ABC transporter was also high in leaves, stem and roots of rice-tartary suggesting that this gene plays key role in the transport of flavonoids (rutin, quercetin and quercitrin) in *F. tataricum*. It has also been shown that biosynthesis of flavonoids takes place in lower parts of *Fagopyrum* spp. and then gets transported to upper parts (Li et al., 2010).

Significantly higher expression of 4 TDFs encoding for ubiquitin protein ligase, ABC transporter, sugar transporter and calmodulin binding protein in developing seeds (S7 stage) of *F. tataricum* compared to *F. esculentum* suggests their major involvement in nutritional superiority as well as unique seed morphology of rice-tartary buckwheat. The calmodulin

binding proteins regulate diverse cellular processes by interacting with other proteins and help in secondary metabolism by acting as a secondary messengers through signal transduction (Zielinski 1998, Luan et al., 2002). In addition, calmodulin proteins are also known to induce anthocyanin biosynthesis in *V. vinifera* (Vitrac et al., 2000). Extensins, the major structural proteins in plant cell wall play important role in various biological processes such as embryo development, root hair growth, seed coat development, defense etc. (Lianhua et al., 2010; Xu et al., 2011). The higher expression of extensin protein (JQ003863) in mature seeds of rice-tartary buckwheat in comparison to common buckwheat has been observed.

Transcription factors are known to play important role in various seed development process and regulation of secondary metabolism (Karin 1990; Fujimoto et al., 2000; Agarwal et al., 2011). Zn finger binding proteins (JN982723) have been implicated in regulation of important biological processes such as flower and seed development, seed germination, stress tolerance in *Arabidopsis* (Papi et al., 2000; Xu and Li 2003). In our study, the expression of TDF encoding Zn finger binding protein was relatively higher in seed developing stages S8 and S9 of common buckwheat in comparison to rice-tartary buckwheat suggesting its contribution in morphological and nutrition differences in seeds of *Fagopyrum* species as the level of its expression was negligible in leaves, stem and roots. The transcript of a TDF encoding for another transcription factor MYB 118 (JN982734) was high in the flowers (S6) of rice-tartary compared to common buckwheat which is unique to this study because *AtMYB118* was earlier reported to play important role in embryogenesis and seed development & maturation in *Arabidopsis* (Wang et al., 2009b; Zhang et al., 2009).

The ubiquitin protein ligases (JN982742) are known to regulate various biological processes like photomorphogenesis, hormonal response, senescence, floral, embryo and seedling

development through degradation of proteins as well as regulating phenylpropanoid pathway during UV stress and sugar signaling during seedling development. (Endt et al., 2002; Peng et al., 2003; Huang et al., 2011). Relatively higher expression of Ub protein ligase in immature seeds (S7) of rice-tartary buckwheat might be contributing to differences in seed morphology of both the *Fagopyrum* species.

Significantly higher expression of most of the selected TDFs during early seed formation stage (S7) of *F. tataricum* reflects their biological importance in maintaining higher amounts of rutin, which otherwise drops significantly in the same stage of *F. esculentum*. Therefore, in addition to structural genes, the other classes of genes such as regulators, modifiers and transporters are equally important in contributing to higher flavonoids content. The upregulation of selected TDFs in *F. tataricum* during early seed maturation stage (S7) i.e. the transition from flowers to seed formation also reflects their contribution in not only higher rutin content but also in other biological process which are contributing to overall nutritional and morphological differences between both the *Fagopyrum* species.

Summary

Buckwheat has several medicinal and nutritional values due to the favorable composition of protein complex with high content of lysine, fibrous material, mineral compounds, vitamins, fagopyritols, bioflavonoid rutin and also an important gluten-free crop. Out of 20 species in the genus *Fagopyrum* only two, *Fagopyrum tataricum* (tartary buckwheat) and *F. esculentum* (common buckwheat) are mainly cultivated in North-Western and North-Eastern Himalayas of India. The fruits of common buckwheat are triangular, large 3 edged achene in comparison to tartary buckwheat where fruits are small, ovoid, and conical with dull irregular faces on each side. Buckwheat is known as the richest source of rutin with 50x higher rutin content in tartary buckwheat (0.8-1.7% D.W.) in comparison to common buckwheat (0.01% D.W.). In addition, fagopyritols (mono-, di- and trigalactosyl derivatives of D-chiro-inositol) content is also high in tartary buckwheat (40%) compared to common buckwheat (21%) thus, helps in the treatment of diabetes. Therefore, tartary buckwheat is considered an excellent food material with a potential for preventive nutrition. But tartary buckwheat has a tightly adhering hull that makes it difficult to dehull. However, Rice-tartary is a type of tartary buckwheat (*F. tataricum*) with a non-adhering hull property and can be a potential nutraceutical food source. Consequently, overall nutritional superiority of *F. tataricum* seeds coupled with the existence of non-adhering hull phenotypes with ease in processing suggests that molecular basis of morphological and biochemical differences in the seeds of two *Fagopyrum* species need to be deciphered.

Estimation of rutin content in 195 accessions of *F. tataricum* collected from North-Western Himalayas of India showed significant variation for rutin content (6 µg/mg- 30 µg/mg). *F. tataricum* accessions IC-14889, IC-42421 and IC-329457 contained maximum rutin content

with 30.21µg/mg, 24.91µg/mg and 24.30µg/mg respectively. Efforts have been made to correlate DNA fingerprinting profile with the high and low rutin content in *F. tataricum* accessions could be helpful in the conservation of germplasm. AFLP profiling of high and low rutin content accessions of *F. tataricum* was undertaken with 19 AFLP primers combinations (*Eco*RI/*Mse*I). In total 907 clearly visible fragments were detected out of which 136 were found to be polymorphic with an average of 7.15 polymorphic fragments per primer combination. The hierarchical and model-based cluster analyses of AFLP data strongly suggested that the 18 sampled populations of *F. tataricum* were clustered into two separate groups. Cluster I was divided further into 3 clusters wherein, cluster-Ia contained all low rutin content accessions (IC-49676, IC-310046, IC-170583, IC-18664 and EC-99945); cluster-Ib contained high rutin accessions of tartary buckwheat (IC-26756, IC-107962) and cluster-Ic contained high rutin accessions of rice-tartary buckwheat (IC-329457, IC-329456 and IC-258233). Cluster II contained all high rutin content accessions (IC-14253, EC-18282, IC-274331, IC-49667, IC-14889, IC-313136, IC-310045 and IC-42421). Therefore, on the basis of AFLP profiling, high and low rutin accessions were grouped separately.

Understanding the molecular basis of nutritional and seed component differences between tartary and common buckwheat would be helpful in exploring novel genes/alleles contributing to higher flavonoid content in tartary buckwheat. Physiological and molecular dissection of higher rutin content in *F. tataricum* compared to *F.esculentum* and its correlation with the expression of flavonoid pathway genes was carried out. The analysis of rutin content dynamics at different growth stages, (from seed germination to mature seed formation) of *Fagopyrum* species revealed that rutin content was highest in the inflorescence stage of both the species with 15.75 µg/mg f.w. in IC-329457 compared to IC-540858 (10.42 µg/mg f.w.) and IC-

14889 (10.26 µg/mg f.w.). The rutin content then decreased during post-flowering stages, S7 & S8 (immature seeds) with a sharp decrease in common buckwheat compared to tartary and rice tartary buckwheat. The rutin content was ~17 folds higher at stage S7 in IC-14889 and ~31 folds higher in IC-329457 compared to IC-540858. The increase in rutin content was highest at mature seeds (S9) of *F. tataricum* with ~55 folds in IC-14889 and ~43 folds in IC-329457 compared to IC-540858 of *F. esculentum.* Therefore, it has been concluded that relatively higher biosynthesis and accumulation of flavonoids (rutin) takes place during post-flowering stages of *F. tataricum* compared to *F. esculentum.*

Higher nutritional profile of seeds of tartary buckwheat compared to common buckwheat, particularly the rutin content which is almost same till flowering stages of both the species but then increases significantly during post flowering stages in tartary buckwheat was found to be encouraging. The lack of basic genome resources such as genetic map, ESTs, genome libraries, molecular markers, etc. further complicated molecular dissection of flavonoid machinery in buckwheat. The cloning and identification of flavonoid (rutin) biosynthetic pathway genes in buckwheat was done through comparative genomics via retrieval of structural genes of flavonoid biosynthesis from different plant species followed by designing and amplification of conserved primers on *Fagopyrum* species. The fragments of flavonoid pathway genes PAL, 4CL, C4H, CHI, F3H, FLS and F3'H genes were isolated. The fragment size of PAL was 1.14 kb whereas for C4H, 4CL, F3H, F3'H and FLS genes fragment sizes ranged from 180-350 bp. The gene fragments for PAL, C4H, FLS F3H showed 70-95% identity with the corresponding genes from other species, whereas gene fragments for F3'H, 4CL and CHI showed 50-70% of identity. The expression analysis, through real time RT-PCR, in different growth stages of *Fagopyrum* species with gene specific primers revealed that transcripts of PAL, CHI, FLS and CHS were relatively

higher in rice-tartary buckwheat compared to common buckwheat. In conclusion, the positive correlation of 4 flavonoid pathway genes, PAL, CHS, CHI and FLS with the rutin content variation at different growth stages of *Fagopyrum* species was observed.

In addition to the contribution of flavonoid pathway structural genes in biosynthesis of higher rutin in *F. tataricum*, differential transcript profiling was undertaken to determine, what other genes (transcription factors, modifiers, transporters etc), are contributing to higher amounts of rutin content during post flowering stages of *F. tataricum*. It has been suggested that the expression of one or more than one flavonoid pathway genes is regulated by different classes of transcription factors, mediating either an increase or decrease in the accumulation of transcript of one or more than one gene of flavonoid biosynthetic pathway. Analysis of differentially expressed genes during different seed developmental stages (flower to mature seed formation) helped to identify genes contributing to nutritional and morphological differences between rice-tartary and common buckwheat. Differential transcript profiling through cDNA-AFLP resulted in the identification of total of 509 differentially expressed transcripts (TDFs) out of which 167 were eluted from the gel based on presence/ absence or differences in level of expression, sequenced and annotated for putative biological function through BLASTN and BLASTX in GenBank. Each sequence was functionally annotated through literature survey and the TAIR database. The TDFs represented genes controlling different biological processes such as general and secondary metabolism (33%), regulation (18%), signal transduction (14%), transportation (13%), cellular organization (10%), transposable elements (7%), photosynthesis (4%) and defense & response to stimuli (1%). Most of the TDFs encoding for transporters, transcriptional regulation, secondary metabolism, photosynthesis & energy, carbohydrate, protein and amino acid metabolism showed relatively higher expression pattern in *F. tataricum* compared to *F.*

esculentum. In order to understand the involvement of TDFs in flavonoid content differences between two *Fagopyrum* species, TDFs with putative roles in transport, regulation, metabolism and energy were selected for real time RT-PCR analysis in flower to mature seed stages of both the species as well as vegetative tissues (leaf, shoot and root stages). The quantitative RT-PCR analysis of nine TDFs representing biological functions in regulation, metabolism, signaling and transport of secondary metabolites showed that all TDFs (Ubiquitin protein ligase, ABC transporter, sugar transporter) except MYB 118 showed significantly higher expression in early seed formation stage (S7) of *F. tataricum* compared to *F. esculentum*. The up-regulation of TDFs during transition from flowering to seed formation suggests their involvement not only in the higher rutin content of *F. tataricum* over *F. esculentum* but also in other biological processes contributing to overall nutritional and morphological differences in the seeds of both the species.

The present study concludes that in addition to structural genes, the other classes of genes such as regulators, modifiers and transporters are equally important in contributing to higher flavonoids content. The identification of several genes representing regulators, modifiers or transporters, has opened up avenues to investigate their precise role in contributing to higher rutin content as well as overall nutritional superiority of *F.tataricum* over *F. esculentum*. The TDFs with differential expression patterns can be tested through gene silencing or over-expression to ascertain their role in rutin biosynthesis or seed maturation in *Fagopyrum* species. Full length cloning of informative TDFs coupled with the cloning of promoter regions for those genes would provide insights into what determines differential expression of TDFs between *Fagopyrum* species. The candidate gene sequences for these genes can also be utilized either in the development of gene markers or for metabolic engineering for genetic improvement of *Fagopyrum* species for higher flavonoid (rutin) content.

List of Publications from PhD research work

A) Research Papers in International Journals

1. Gupta N, Sharma S, Rana JC and Chauhan RS (2011) Expression of flavonoid biosynthesis genes vis-à-vis rutin content variation in different growth stages of *Fagopyrum* species. J Plant Physiol. 168: 2117-2123
2. Gupta N, Sharma S, Rana JC and Chauhan RS (2011) AFLP fingerprinting of tartary buckwheat accessions (*Fagopyrum tataricum*) displaying rutin content variation. Fitoterapia 83:1131-1137
3. Gupta N, Naik PK and Chauhan RS (2011) Differential transcript profiling through cDNA-AFLP unravels complexity of rutin biosynthesis and accumulation in seeds of a nutraceutical food crop (*Fagopyrum* spp.). BMC Genomics 13:231

B) Review article and Book chapter

1. Panwar A, Gupta N and Chauhan RS (2012) Biosynthesis and accumulation of flavonoids in *Fagopyrum* spp. *Eur. J Plant Sci. Biotech* 6:17-26
2. Rana JC, Chauhan RS, Sharma TR and Gupta N (2012) Analyzing problems and prospects of buckwheat cultivation in India. *Eur. J Plant Sci. Biotech* 6:50-56
3. Chauhan RS, Gupta N, Sharma S, Rana JC, Sharma TR and Jana S (2010) Genetic and genome resources in buckwheat- present status and future perspectives. Eur. J Plant Sci. Biotech. 4:33-44.
4. Chauhan RS, Gupta N, Sharma S and Rana JC (2009) Development of genome resources and understanding molecular biology of rutin biosynthesis in a nutraceutical food crop (buckwheat) of high altitudes. Advances in Agriculture Environment and Health: Fruits, Vegetables, Animals and Biomedical Sciences (Singh et al. Eds. 2009) pp 323-334.

C) International Conferences

1. Chauhan RS, Gupta N, Panwar A and Rana JC (2011) Molecular dissection of flavonoids (rutin) biosynthesis in a nutraceutical food crop (*Fagopyrum* spp.). Emerging Trends on Food and Health Security in Cold Dessert (23-25 Sept.), DIHAR, Leh.

2. Gupta N, Sood P and Chauhan RS (2011) Comparative & functional genomics towards gene discovery in a nutraceutical food crop, buckwheat (*Fagopyrum* spp.). International Plant & Animal Genomes Conference (15-19 Jan.), San Diego, California, USA.
3. Gupta N, Sharma S and Chauhan RS (2010) Comparative genomics to elucidate rutin biosynthesis genes in a nutraceutical food crop, *Fagopyrum tataricum*. International Conference on Genomics Sciences- Recent Trends (ICGS 12-14 Nov.), Madurai Kamraj University (TN), India.
4. Gupta N, Sharma S, Rana JC and Chauhan RS (2008) Comparative genomics towards understanding molecular biology of rutin biosynthesis in a nutraceutical food crop, *Fagopyrum tataricum*. International Conference on Molecular Biology and Biotechnology (ICMBB 19-21Oct.), Banasthali Vidyapith (Rajasthan), India.

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