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REVIEW

# Physiological Roles of Rutin in the Buckwheat Plant

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## Abstract

The buckwheat plant contains high levels of rutin (flavonol 3-*O*-rutinoside) in many organs, including its seeds, cotyledon, leaves, stem, and flowers. The enzymes that catalyze the decomposition and synthesis of rutin in buckwheat are unique in terms of having relatively low $K_m$ values, indicating that buckwheat developed rutinosidase and glycosyl transferase enzymes specifically suited for rutin metabolism. In Tartary buckwheat seeds, high levels of rutin content and rutinosidase activity cause strong bitterness, which may effectively protect the seeds from being eaten by animals. The stress responses observed in buckwheat leaves suggests that rutin and rutinosidase are involved in enhancing the defense system against environmental stresses, including UV light, low temperature, and desiccation.

**Discipline:** Plant protection
**Additional key words:** rutinosidase, stress, Tartary buckwheat, quercetin

## Introduction

Rutin is a glycoside of flavonoid widely distributed among plantae (Sando & Lloyd 1924, Couch et al. 1946, Haley & Basin 1951, Bandyuko & Sergeeva 1974, Fabjan et al. 2003). Buckwheat also contains high level of rutin in nearly all organs, including seeds, cotyledons, leaves, stems, and flowers (Kalinova & Dadakova 2006). Accordingly, buckwheat has been utilized as a rutin-rich food material and processed into various foods (Ikeda 2002, Kreft et al. 2006). In Japan, buckwheat is cultivated, not only for use in traditional foods, but also as an ingredient for health foods, because it contains several bioactive compounds such as rutin. Rutin benefits human health in several ways: strengthening fragile human capillaries (Griffith et al. 1944, Shanno et al. 1946); antioxidative activity (Afanas'ev et al. 1989, Afanas'ev et al. 2001, Jiang et al. 2007, Awatsuhara et al. 2010); antihypertensive activity (Matsubara et al. 1985); anti-inflammatory activity (Afanas'ev et al. 2001); and alpha-glucosidase inhibitory activity (Li et al. 2009).

From a plant growth perspective, plant flavonoids act to reduce environmental stress; e.g. via UV-B screening, antioxidant activity, and disease resistance (Harborne & Williams 2000).

There are two major cultivated species of buckwheat: common (*Fagopyrum esculentum* Moench) and Tartary buckwheat (*Fagopyrum tataricum* Gaertn.). Although the cotyledons and leaves of both contain considerable rutin, concentrations in the seeds of Tartary buckwheat are approximately 100-fold higher than those of common buckwheat. In addition, Tartary buckwheat seed also contains high levels of rutinosidase activity (Yasuda et al. 1992, Yasuda & Nakagawa 1994, Suzuki et al. 2002). Based on these properties, Tartary buckwheat represents a good model to study the physiological roles of rutin in buckwheat seeds.

In this review, we summarize the possible physiological roles of rutin in buckwheat, focusing on the accumulation patterns and tissue-specific distribution of rutin during plant development, particularly during seed ripening, germi-

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Abbreviations: DAG; days after germination, f3g; flavonol 3-glucosidase, GT; glucosyltransferase, RDE; rutin-degrading enzyme, RT; rhamnosyltransferase, TDP; thymidine diphosphate, UDP; uridine diphosphate, UV; ultraviolet
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nation, and vegetative growth. The first part covers the purification and characterization of enzymes related to rutin metabolism, while the second part describes the accumulation of rutin in organs, including cotyledons, leaves, and seeds, during different developmental stages. The effects of stress on leaf rutin concentrations and rutinosidase activity are also discussed.

## Rutin Metabolism

### 1. Synthesis

Rutin comprises the aglycone quercetin and the disaccharide rutinose and is accordingly assumed to be synthesized via the 3-*O*-glycosylation of quercetin, followed by the rhamnosylation of isoquercitrin (Barber et al. 1963, 1991) (Fig. 1). In many plant species, glucosylation of flavonoids is catalyzed by flavonoid glucosyltransferases such as UDP-Glc: flavonoid 3-O-glucosyltransferases (3GT). 3GT has been the most well-studied glucosyltransferase in maize (*Zea mays* L.) (Futtek et al. 1988), barley (*Hordeum vulgare* L.) (Wise et al. 1990), and grape (*Vitis vinifera* L.). In common buckwheat, 3GT has only been characterized in cotyledons, from which it was purified 171-fold to homogeneity (final specific activity of 1.46 pkat per mg protein) (Suzuki et al. 2005a).

The molecular weights of 3GT of common buckwheat are 56,000 and 58,600, as estimated by SDS-PAGE and gel filtration, respectively, suggesting that common buckwheat 3GT is a monomer, like other 3GTs, while the optimal pH for 3GT activity is around 7.0 and substrate specificity for the sugar acceptor of common buckwheat 3GT varies among flavonoids. The lowest reported $K_m$ is 27 μM for quercetin. Compared to quercetin, common buckwheat 3GT has at least six-fold lower specificity for apigenin, kaempferol, luteolin, and naringenin. This substrate affinity profile contrasts to that of grape 3GT, which displays a high affinity; not only for quercetin, but also other flavonols, including kaempferol (Ford et al. 1998).

With respect to sugar donors, common buckwheat 3GT has the lowest $K_m$ for UDP-Glc (1.04 mM). This $K_m$ value resembles those of other 3GTs, such as grape, which also has a $K_m$ of 1.04 mM for UDP-Glc (Ford et al. 1998). Barber (1963) reported that both TDP-Glc and UDP-Glc are suitable sugar donors for rutin biosynthesis in the mung bean (*Phaseolus aureus* Roxb.), although the $K_m$ value for TDP-Glc markedly exceeded that of UDP-Glc. Watanabe and Ito (2002) reported that common buckwheat seedlings contain several C-glycosyl flavonoids, including apigenin-8-C-glucoside, apigenin-6-C-glucoside, luteolin-8-C-glucoside, and luteolin-6-C-glucoside. These flavonoid compounds are synthesized just after germination, and gradually decline thereafter. In contrast, the rutin concentration increases as the plant develops.

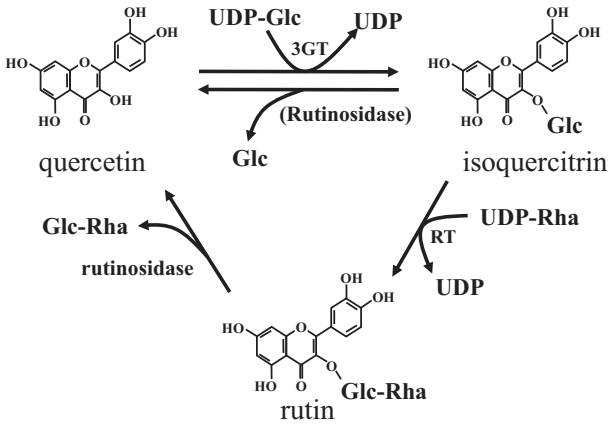


**Fig. 1. Catalysis of rutin synthesis and decomposition**
3GT: flavonol 3-*O*-glycosyltransferase, RT: rhamnosyltransferase

Understanding the mechanisms of rutin synthesis will mean examining the characterization of rhamnosyltransferase (RT) activity, which catalyzes the final step in rutin synthesis. Furthermore, the relationship between 3GT expression and RT activity, and its role in controlling rutin biosynthesis should be clarified, despite the lack of reports on RT characterization. However, in flowers of common and Tartary buckwheat, cDNAs with high homology to plant rhamnosyltransferases have been identified in *de-novo* sequencing analyses using next-generation sequencers (Logacheva et al. 2011). Our research group also obtained cDNAs from Tartary buckwheat root with high homology to RT. In addition, a few studies have reported the expression of mRNAs related to the rutin biosynthesis pathway (Kim et al. 2013a, 2013b, Thwe et al. 2013). The information provided by these studies will aid future investigations into breeding rutin-rich varieties of Tartary buckwheat.

### 2. Decomposition

The characterization of enzymes involved in rutin decomposition is also important to understand the roles of rutin in Tartary buckwheat. Rutinosidase catalyzes the hydrolysis of the 3-glycoside unit of flavonols, which is speculated to be the precursor of rutin (Barber 1963, Barber & Behrman 1991) (Fig. 1). Rutinosidase activity has been detected in a number of plants (Suzuki 1962, Yasuda & Nakagawa 1994, Suzuki et al. 2002, Baumgertel et al. 2003) and microorganisms (Hendson et al. 1992, Narikawa et al. 2000). The first report on Tartary buckwheat rutinosidase was an RDE purified from seeds (Yasuda & Nakagawa 1994), while the second was f3g and also purified from seeds (Suzuki et al. 2002). RDE and f3g have similar char-

acteristics, with the exception of molecular weight and $K_m$, and comprise at least two major isozymes. The molecular weights of the two identified f3g isozymes are 58,200 (f3g I) and 57,400 (f3g II) on SDS-PAGE, and 89,000 for both isozymes on gel filtration. The optimal pH, temperature, kinetic constants and $V_{max}$ for rutin and isoquercitrin are also highly similar for both f3g isozymes. Although the optimal pH and temperature resemble those of RDE (Yasuda & Nakagawa 1994), the kinetic constants (RDE: $K_m$ values for rutin are 120 and 130 mM respectively for each of the isozymes), while the molecular weights (RDE: 68,000 on SDS-PAGE and 70,000 by gel filtration) of the latter differ markedly.

The sequences of the amino terminus of both f3g isozymes are identical within the first 15 residues of f3g I and the first 10 residues of f3g II), and share an identity with other glycosidases, such as cyanogenic-beta-glucosidase (*Trifolium repens* L.) and thioglucosidase (*Arabidopsis thaliana* [L.] Heynh.). F3g catalyze the hydrolysis, not only of rutin, but also isoquercitrin, although the $V_{max}$ value for isoquercitrin is only one-tenth of that for rutin, suggesting that isoquercitrin is the precursor to rutin (Suzuki 1962, Barber & Behrman 1991). The finding that f3g catalyzes the hydrolysis of both rutin and isoquercitrin may relate to the catalytic control of rutin levels in the Tartary buckwheat plant.

Regarding the physiological role of rutin in common and Tartary buckwheat plants, Afanas'ev et al. (2001) showed that rutin complexed with transition metals efficiently scavenges free radicals *in vitro*. Another possible role of rutin relates to the anti-fungal activity of its aglycone quercetin component. It was reported that the anti-fungal agent 3, 4-dihydroxybenzoic acid is formed when onion scale leaves brown due to the peroxidase-dependent oxidation of quercetin (Takahama & Hirota 2000). As common and Tartary buckwheat also exhibits peroxidase activity in seeds (Kondo et al. 1982, Suzuki et al. 2005c, 2006, 2010, 2012) and leaves (Mikami et al. 2013), f3g might catalyze the first step in producing an anti-fungal agent in seeds during germination.

## Rutin and rutinosidase activity in seeds, cotyledons and the leaves of common and Tartary buckwheat

To further investigate the physiological roles of rutin in common and Tartary buckwheat, we monitored the accumulation and tissue-specific distribution patterns of rutin during Tartary buckwheat development (Suzuki et al. 2005b, 2009). In addition, because enzymes related to rutin biosynthesis and decomposition also influence the function of rutin, we investigated their activities during the developmental stages of germination (cotyledon), leaf expansion, and seed ripening in common and Tartary buckwheat.

### 1. Seeds

Several reports have examined rutin concentrations and rutinosidase activities in seeds of common and Tartary buckwheat (Kitabayashi et al. 1995ab, Yasuda & Nakagawa 1994, Ohsawa & Tsutsumi 1995, Suzuki et al. 2002, Morishita & Tetsuka 2002, Végvári et al. 2008). The rutin concentration in Tartary buckwheat seeds ranges from 1,100 to 1,950 mg/100 g dry weight, which is two orders of magnitude higher than that found in common buckwheat. Rutin content in common and Tartary buckwheat seeds increases after pollination and peaks in mature seeds (several days after pollination (Jianchun & Yu 1992, Suzuki et al. 2002). Jiang et al. (2007) investigated the rutin levels of three buckwheat species, common buckwheat, Tartary buckwheat, and *F. cymosum*, and concluded that seed rutin plays an important role in antioxidant activity. During seed ripening, rutin concentrations, rutinosidase activity, and 3GT activities increase in seeds (Suzuki et al. 2002). The rutinosidase activity in Tartary buckwheat seeds was sufficient to hydrolyze considerable rutin in the seeds within a few minutes. In Tartary buckwheat seeds, rutin is mainly distributed in the embryo, whereas nearly all rutinosidase activity occurs in the testa. Therefore, rutin in embryos is physically separated from rutinosidase activity by differences in organ distribution, a finding that matches a report by Mukasa et al. (2009).

In embryos, cells actively divide during seed development. In such seeds, free radical- or UV light-induced DNA damage significantly influences plant development. We have also shown that the levels of rutin and rutinosidase in Tartary buckwheat seeds influence the taste of seeds. Tartary buckwheat is traditionally known as 'bitter buckwheat' because the Tartary buckwheat flour grain is strongly bitter. From these findings, we recently developed a new variety of Tartary buckwheat with rutinosidase activity approximately two orders of magnitude lower than that of ordinary varieties. Analysis using this trace-rutinosidase variety has shown a relationship between the levels of rutin hydrolysis and bitterness. As bitter foods are widely hated by humans and animals (Drewnowski et al. 2000, Drewnowski 1997, Hladik 1996), rutin and rutinosidase activity may be involved in plant defense against being eaten.

### 2. Seedlings

The rutin concentration changes as common buckwheat seedlings grow (Troyer 1955, Watanabe & Ito 2002, Kim et al. 2004, Kim et al. 2006, 2007, Krahl et al. 2008). In seedlings, flavonoids other than rutin, including orientine and isovitexine, have also been identified. Suzuki et al. (2007) investigated changes in rutin concentration, in addition to rutinosidase and 3GT activities, during the seedling

growth of Tartary buckwheat, and found that the rutin concentration gradually increased from 0 to 12 DAG. Mature seeds also contain considerable rutin (approximately 2% of the dry weight), indicating that Tartary buckwheat cotyledons comprise approximately 1.25% rutinose as rutinoside, which may serve as a source of carbohydrate nutrition during germination and seedling growth. However, the rutin concentration in Tartary buckwheat cotyledons increased following germination, indicating that rutin in Tartary buckwheat cotyledon is not used as a nutritional source during germination and cotyledon growth.

Rutin was shown to deter larval feeding by certain species of insects (Simmonds 2003). In our study (Kim et al. 2006), as rutin concentrations in mature cotyledons were relatively high, representing approximately 4% of the dry weight, rutin may have a role in preventing damage from insects. In common buckwheat, rutin concentrations increase as the seedling develops. For example, from 3 to 5 DAG, the rutin concentration of cotyledons exposed to light exceeded that of those grown in darkness (Kim et al. 2006), while the rutin concentration in mature cotyledons (5 DAG) was roughly 4% of the dry weight. The highest rutin concentration in cotyledons grown under light conditions emerged at 3 DAG, whereas the concentration peaked at 4 DAG under dark conditions and did not diminish until 7 DAG under light and dark conditions respectively. In contrast, the 3GT activity began to increase just after germination, peaked at 4 DAG, and then rapidly decreased until 7 DAG. In this study, in these cotyledons, more than 50% of the rutin was observed in the upper epidermis of the cotyledons.

Several studies have demonstrated how rutin functions as a UV filter under light irradiation (Suzuki et al. 2002, Margna et al. 1990, Suzuki et al. 2005b). However, both rutin concentration and 3GT activity were even elevated in common buckwheat cotyledons grown in darkness. This finding suggests that in addition to UV screening, rutin may have other roles, such as enhancing the defense system against cold or desiccation stress in Tartary buckwheat leaves (Suzuki et al. 2005b), as described in the next section.

Suzuki et al. (2005a) also found that rutinosidase activity, which is localized in the testa, begins to decrease immediately after germination. At 4 DAG testae contained 80% of the rutinosidase activity compared to the testa at 0 DAG. From 0 to 4 DAG, the testa adheres to the cotyledon and husk. From 5 DAG, both testa and husk are easily separated from the cotyledon, which exhibits relatively high surface rutinosidase activity (approximately 25% of that of the testa). The rutinosidase enzymes found on the cotyledon surface were likely exported from the testa because the cotyledon itself contained little rutinosidase activity compared with the cotyledon surface. In contrast, rutin was predominantly distributed in the epidermis of the cotyledon, where it may be hydrolyzed to quercetin by rutinosidase located at the cotyledon surface if the cotyledon is damaged. When onion scale leaves brown, the anti-fungal agent 3,4-dihydroxybenzoic acid is formed by the peroxidase-dependent oxidation of quercetin (Takahama & Hirota 2000). The rutinosidase activity on the cotyledon surface may play a similar anti-fungal role. Based on these results, rutin and rutinosidase activity in Tartary buckwheat appear to have different physiological roles during cotyledon growth compared to those in common buckwheat.

**3. Leaves**

Common and Tartary buckwheat leaves contain rutin levels similarly high to those found in cotyledons. In other plants, flavonoids are mainly located in the epidermis of leaves (Harborne & Williams 2000). Rutin accumulation has also been detected in the leaves of common and Tartary buckwheat (Zhanaeva 1996, Kitabayashi et al. 1995ab). Suzuki et al. (2005b) examined the rutin concentration, and rutinosidase and 3GT activities in different stages of Tartary buckwheat leaves(L1=cotyledon, L2= senescent leaf, L3-L6=mature leaf, and L7 and L8=young leaf) and found that the rutin concentration peaked in L7 leaves and decreased with increasing leaf age, reaching almost zero in yellow senescent leaves. The rutin concentration on a dry-weight basis peaked in L8 leaves (>20% of the dry weight), the youngest, and gradually decreased with increasing leaf age. These results effectively match the report of Zhanaeva (1996), who found that the rutinosidase activity on a dry-weight basis was higher in young leaves of common buckwheat, peaked in L6 leaves, and then gradually decreased. The 3GT activity showed a similar pattern to that of rutin levels, suggesting that 3GT plays a role in rutin synthesis.

Rutin is mainly located in the epidermis of Tartary buckwheat leaves. This localization matches a report by Zhanaeva (1996) on common buckwheat leaves and by Harborne and Williams (2000) on the leaves of other plant species. Notably, more than half the rutin in Tartary buckwheat leaves is located in the upper epidermis, which reinforces the idea that rutin plays a role in UV screening.

We have also investigated the effects of stress on rutin synthetic and degradative enzymes in Tartary buckwheat leaves. An increased level of ambient UV radiation can adversely affect the growth of common buckwheat (Mateja & Barbara 2007, Ozbolt et al. 2008, Yao et al. 2006) and increase leaf rutin concentrations. Kreft et al. (2002) showed that after long-term UV-B radiation, reducing the UV-B radiation level resulted in a lower rutin concentration in common buckwheat leaves and flowers than that found in plants continually exposed to ambient UV-B levels. In a field test conducted by Yao et al. (2006), the rutin concentration in leaves was increased by supplemental UV-B radia-

tion. These findings support the idea that rutin serves as a UV screen.

To confirm the role of rutin as a UV screen, Suzuki et al. (2005b) performed stress treatments on Tartary buckwheat leaves using an experimental protocol designed to decrease experimental error due to differences in the growing stage or individual plant characteristics (Fig. 2). At 28 DAG, L7 leaves of field-grown Tartary buckwheat were harvested, and individual leaves were subjected to both stress and control treatments to minimize variability. Measurements of rutin concentration and rutinosidase activity revealed these parameters did not change immediately after stress treatments. However, the rutin concentration increased significantly with UV-B radiation or desiccation treatment, but was not markedly altered by cold treatment.

Many studies have reported that flavonoids function as a UV screen (Harborne & Williams 2000). These observations are consistent with the increased rutin concentration found in common buckwheat leaves exposed to UV-B radiation. In common buckwheat, long-term UV-B radiation results in an increase in UV-B absorbing compounds (Kreft et al. 2002). Rutin concentration is affected by humidity and irrigation and peaks in common buckwheat plants cultivated under dry conditions (Ghouzbdi et al. 2009), meaning it may play a protective role against desiccation. Rutinosidase activity was also shown to increase significantly in response to various stress treatments; a 363% increase was observed under UV-B radiation, 190% under cold treatment (-5 °C, 5 minutes), and 158% in response to desiccation, as compared to the control (Suzuki et al. 2005b). After UV-B radiation, the rutin concentration and rutinosidase activity increased concurrently.

The increased rutinosidase activity results in an increase in quercetin and rutinose concentrations, and may serve to supply quercetin as a peroxidase substrate. Under stress conditions, peroxidase plays an important role in defending plants against oxidative damage (Kolattukudy et al. 1992, Bradley et al. 1992). Because quercetin is a suitable substrate for guaiacol peroxidase (Amako et al. 1994), quercetin, which is produced from rutin, may be used as a substrate of guaiacol peroxidase to prevent oxidative damage to Tartary buckwheat leaves. This reinforces the concept that the stability of rutin against oxidative degradation far exceeds its aglycone quercetin (Afanas'ev et al. 1989).

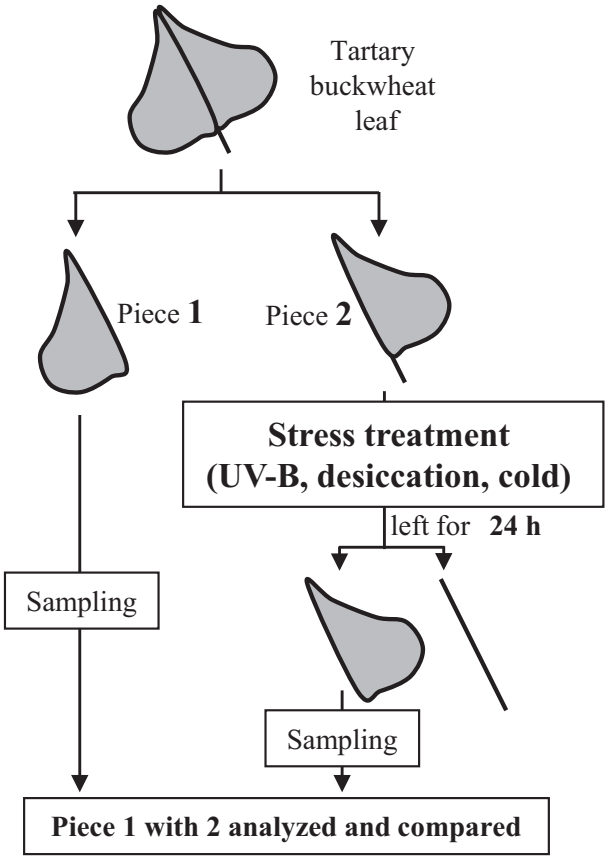


**Fig. 2. Flowchart of environmental stress treatments in the study be Suzuki et al. (2005b)**

An untreated leaf was used as a control. The stress-treated leaf was left for 24 h at 22°C with adequate water. The rutin concentration and rutinosidase activity were measured after the main vein had been removed and the results of pieces 1 and 2 were compared (value of piece 1 of each sample = 100).

## Future perspective

From the results described in this review, several possible roles of rutin in common and Tartary buckwheat are described, in protecting against UV and enhancing defense mechanisms against stress. Further elucidating this mechanism would be useful, not only for common and Tartary buckwheat but also other crop breeding to enhance stress tolerance, which will be promoted by the development of mutants with impaired rutin biosynthesis and/or for which rutinosidase activity is necessary.

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