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| 学位論文題目 | ROLES OF RUTIN IN BUCKWHEAT PLANT AND FLOUR<br>（ソバ植物体とソバ粉におけるルチンの役割に関する研究） |
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# 論 文 内 容 要 旨

## Chapter 1. Introduction

Buckwheat (*Fagopyrum esculentum* Moench) is grown in many countries, and is the only known cereal to contain rutin (a kind of flavonoid) in its seeds. To date, several studies have sought to understand the physiological roles of rutin, including as an UV-B screen. However, the physiological role of rutin in buckwheat still requires further clarification, requiring an investigation of developmental rutin accumulation patterns and tissue specific distribution of rutin during buckwheat growth. In addition, a study of the enzymes involved in rutin synthesis and decomposition is also required. Towards these goals, in chapter 2, I first purified and characterized enzymes of rutin synthesis and decomposition in buckwheat. In chapter 3, I then measured the concentration of rutin and related compounds at different stages of buckwheat growth and development. Further, I investigated changes in leaf rutin concentration and rutin glucosidase activity when the leaf was subjected to stress. Based on these studies, the physiological roles of rutin are discussed.

In the Japanese food industry, buckwheat flour is mainly used in making noodles. The freshness of buckwheat flour is very important in noodle manufacturing and quality. However, buckwheat flour deteriorates easily. Several reports have shown that, during storage, lipid degradation and oxidation are the main causes of deterioration in buckwheat flour in terms of measurable quality indexes. Therefore, in chapter 4, I first characterized the enzymes assumed to catalyze lipid degradation and oxidation in buckwheat flour. In a next step, the effects of rutin on flour quality deterioration were investigated with the underlying goal of preventing lipid degradation and oxidation in buckwheat flour. The effects of rutin on the activity of enzymes of lipid degradation and oxidation in buckwheat flour were specifically investigated. The importance of rutin in the buckwheat plant and in quality deterioration of buckwheat flour is discussed.

## Chapter 2. Characterization of enzymes involved in rutin synthesis and decomposition

Buckwheat contains rutin not only in its seeds, but also in its cotyledons, leaf, stem and flour. To date, studies have assigned certain physiological functions to plant flavonoids, namely UV-B screening, antioxidant activity and disease resistance. In buckwheat, several studies have investigated the physiological roles of rutin, but did not sufficient to clarify these roles. Therefore, a study of enzymes involved in rutin biosynthesis and decomposition (Figure 1) was a primary requirement. In this chapter, the enzymes of rutin synthesis and decomposition in common buckwheat and tartary buckwheat [*Fagopyrum tataricum* (L.) Gaertn.] were purified and characterized.

The two monomeric enzymes identified as showing flavonol 3-glucosidase (f3g) activity in tartary buckwheat seeds presented very similar kinetic characteristics. The f3g had high affinity for rutin ($K_m$ ~ 0.12 mM); greater than that for quercetin 3-*O*-glucoside [$K_m$ ~ 1.10 mM] (Table 1). The UDP-Glc: flavonoid 3-*O*-glucosyltransferase (3GT) enzymes in buckwheat cotyledons were monomeric and exhibited a greater affinity for quercetin as a sugar acceptor ($K_m$ ~ 27 μM) than any other flavonoid tested. 3GT showed a higher affinity for UDP-Glc ($K_m$ ~ 1.04 mM) than for other sugar donors (Table 2).

These results suggest that in terms of substrate specificity, f3g and 3GT were adapted to rutin synthesis and decomposition in buckwheat. Therefore, to investigate the physiological roles of rutin, it would be necessary to investigate changes in the contents of rutin and related enzymes of rutin synthesis and decomposition.

## Chapter 3. Changes in rutin content during leaf expansion, grain ripening and cotyledon growth in buckwheat as a function of its enzymic synthesis and decomposition

To further investigate the physiological role of rutin in buckwheat, in chapter 3, changes in the concentration of rutin during buckwheat and tartary buckwheat growth and development, such as in leaves and in the ripening seed and in germinating cotyledons, were investigated. Changes in enzymatic activities related to rutin synthesis and decomposition were also investigated. In addition, changes in rutin concentration and f3g activity in leaves under the various stresses (UV-B radiation, cold and moisture stress) were investigated.

In leaves, rutin concentration and 3GT activity were greatest in the young leaf and then decreased gradually as a function of decreasing leaf position (Figure 2). Rutin was mainly located in the epidermis, particularly the upper epidermis which bore more than half the rutin in the leaf. Several studies have investigated the physiological role of rutin in UV-B screening. Rutin absorbs UV light in the same range as lignin and wax. However in a young leaf, rutin would be more suitable as a UV screening compound than lignin and wax, because young leaves of tartary buckwheat expand very quickly, generally within a few days, and the shape of the leaves changes dramatically. In such leaves, lignin or wax would physically prevent the leaf from expanding whereas rutin would not. These results suggest that compounds used in screening tartary buckwheat leaves' response to UV-B light depend on the leaf's stage of expansion and that rutin is a suitable screening compound during the young stage leaf.

To further clarify the physiological role of rutin by investigating other possible physiological roles of rutin, stress (UV-B, cold, moisture) treatments were imposed on tartary buckwheat leaves. The rutin concentration was increased 122% by UV-B radiation and 129% by moisture stress treatments (Figure 3). On the other hand, f3g activity was increased 363% by UV-B radiation, 190% by cold stress and 158% by moisture stress treatment compared to non-stressed leaves. In addition, the quercetin concentration was increased almost 40-fold UV-B radiation, 240% by cold treatment and 590% by moisture stress treatment, compared to the control. These results also suggest that f3g

activity and rutin in buckwheat have roles other than as a UV screen. The fact that rutin concentration and f3g activity were increased by stress treatment, suggest that rutin and f3g activity may be related to enhancement of the defense system against stress conditions in the tartary buckwheat leaf.

In seeds, rutin concentration increased during seed ripening and remained high in fully ripe seeds (Figure 4). The f3g activity increased together with 3GT activity during seed ripening and remained high with only a slight reduction in fully ripe seeds. In such seeds, the major part of the rutin was found in the embryo, whereas almost all the f3g activity was detected in the testa. In the early stage of seed-development, cells in the embryo divide actively and it undergoes dramatic morphological changes. Damage to DNA from UV light would have a significant deleterious influence on the growth of the embryo or plant. Rutin could be important in the seeds, especially at the early stage of ripening, as a UV-absorbing compound.

In germinating cotyledons of buckwheat (Figure 5), rutin concentration and 3GT activity increased just after germination. The 3GT activity and rutin concentration were high even in buckwheat cotyledons that were grown in darkness and thus were not exposed to UV light. This suggests that rutin in buckwheat cotyledons may have other roles in addition to UV screening. In tartary buckwheat, the f3g activity, which is located mainly in the testa was detected at high levels during cotyledon growth (Figure 6). In addition, the f3g was also present on the surface of the cotyledon. The f3g on the surface of the cotyledon could have been exported from the testa because the cotyledon did not contain much f3g compared with the surface of the cotyledon. On the other hand, rutin was distributed mainly in the epidermis of the cotyledon, suggesting that rutin is hydrolyzed to quercetin by f3g at the surface of the cotyledon if the cotyledon is injured. The f3g activity on the surface of the cotyledon may play a role in producing an anti-fungal agent such as 3,4-dihydroxybenzoic acid, which is formed such as in the browning of onion skin by peroxidase-dependent oxidation of quercetin.

## Chapter 4. Effects of rutin on quality deterioration of buckwheat flour during storage

The freshness of buckwheat flour is important for Japanese buckwheat noodle makers, since buckwheat flour deteriorates easily. Several reports have shown that lipid degradation and oxidation (Figure 7) in buckwheat flour are the main causes of measurable quality deterioration during storage. Therefore, understanding lipid degradation pathways is important in the quality control of buckwheat flour. Rutin may prevent lipid degradation and oxidation because it exhibits antioxidative activity. Therefore, in chapter 4, the effects of rutin on quality deterioration and on the activity of enzymes involved in lipid degradation were investigated. In addition enzymes related to quality deterioration in buckwheat flour were characterized and the effects of rutin on these enzymes were investigated.

To investigate the effects of changes in rutin concentrations, as well as lipase (LIP), lipoxygenase (LOX) and peroxidase (POX) on the quality of buckwheat flour, storage tests of 14 buckwheat varieties for 0, 4, 10 and 30 days at 5°C or 20°C were carried out. There were at least two enzymes of LIP and POX, and two proteins for LOX in buckwheat flour. LIP activity from buckwheat flour was inhibited by rutin whereas POX was not (Figure 8).

During the storage period, the rutin concentration showed negative correlations to water soluble acid (WSA; significant at 30 days of storage (DOS) at 5°C, and after 4 DOS at 20°C) (Table 3). The LIP activity was significantly and negatively correlated to pH positively to WSA. The LOX1 protein concentration showed a negative correlation with WSA (significant at 0 and 4 DOS at 5°C, as well as at 0 and 10 DOS at 20°C). The POX showed a significant correlation to pH and POV at 5°C, whereas this correlation was not significant at 20°C. The rutin concentration showed a negative correlation with to WSA (significant at 30th DOS 5°C, and 4 DOS at 20°C).

From these results, rutin concentration and lipase activity apparently play an important role in lipid degradation and associated quality deterioration of buckwheat flour. To breed a better buckwheat variety, decreasing the LIP activity and increasing rutin concentration in buckwheat seed would be desirable.

## Conclusion

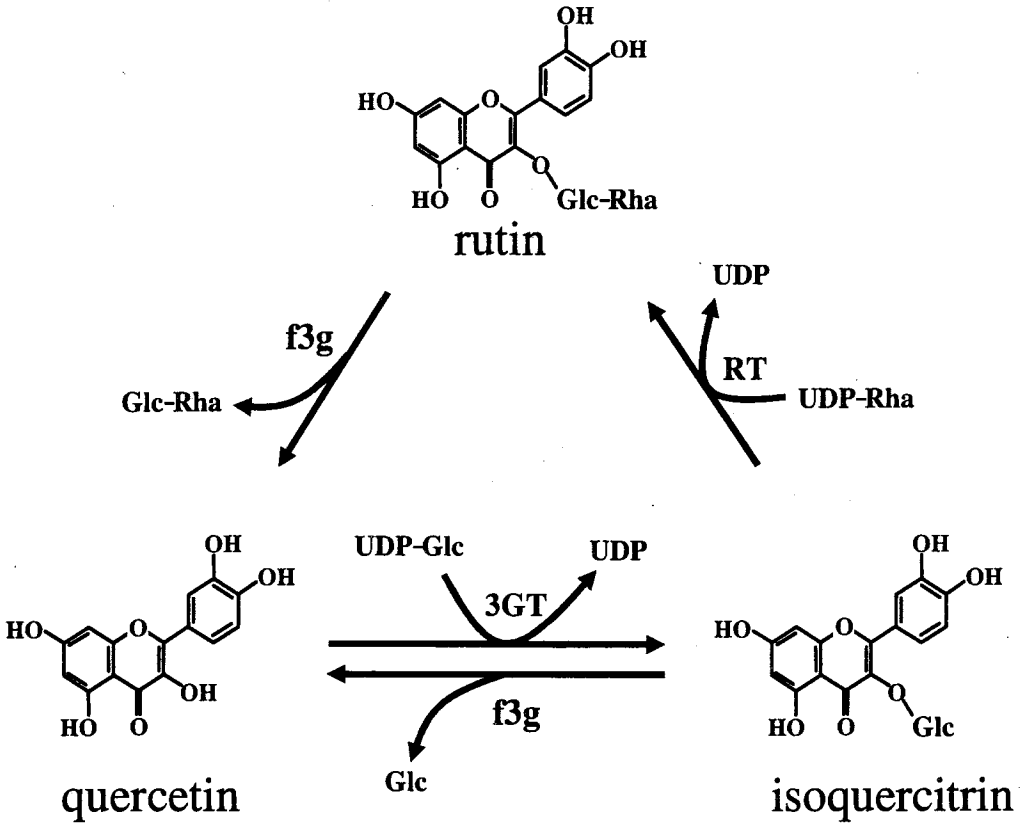
In terms of substrate specificity, f3g and 3GT were adapted to rutin synthesis and decomposition in buckwheat

Changes in the concentration of rutin during buckwheat and tartary buckwheat growth and development, such as in leaves and in the ripening seed and in germinating cotyledons suggest that rutin play a role in UV screening.

The fact that rutin concentration and f3g activity were increased by stress treatment, suggests that rutin and f3g activity may be related to enhancement of the defense system against stress conditions in tartary buckwheat leaf.

Rutin concentration and lipase activity apparently play an important role on lipid degradation and associated quality deterioration of buckwheat flour.

From these studies, it is clear that rutin is a very important compound for the buckwheat plant in terms of its defense system, and also for buckwheat flour in terms of quality maintenance.




f3g: flavonol 3-glucosidase

3GT: flavonol 3-*O*-glucosyltransferase

RT: Rhamnosyltransferase

**Figure 1.** Catalysis of rutin biosynthesis and decomposition.

**Table 1.** Kinetic constants and Molecular Weight of f3g isozymes

| | $K_m$ value[a] | | $V_{max}$ value[a] | | Molecular Weight[b] | |
|---|---|---|---|---|---|---|
| | for rutin (mM) | for isoquercitrin (mM) | for rutin (nkat $mg^{-1}$) | for isoquercitrin (nkat $mg^{-1}$) | SDS-PAGE (kDa) | gel filtration (kDa) |
| f3g I | 0.115 | 1.05 | 609 | 66.3 | 58.2 | 86.0 |
| II | 0.125 | 1.15 | 625 | 68.2 | 57.4 | 86.0 |

[a]Data are means of two measurements. Kinetic constants of purified f3g isozymes for rutin and isoquercitrin were measured under standard assay conditions.

[b]Data are means of three measurements by SDS-PAGE using samples from three independent purifications, and five measurement by gel filtration.

**Table 2.** Substrate specificity of 3GT

| Substrate | Relative activity (%) | $K_m$ mM |
|---|---|---|
| Flavonol | | |
| quercetin | 100.0 [a] | 0.027 |
| kaempferol | 15.2 [a] | n.d. |
| Flavone | | |
| apigenin | 14.9 [a] | n.d. |
| luteolin | 1.0 [a] | n.d. |
| Flavanone | | |
| narigenine | 3.3 [a] | n.d. |
| UDP-Glucose | 100.0 [b] | 1.04 |
| UDP-Galactose | 22.8 [b] | n.d. |
| TDP-Glucose | 17.3 [b] | 25.4 |

[a]: Activity was determined by decrease in substrate concentration in reaction mixture. UDP-Glc was used as a sugar donor.
[b]: Quercetin was used as a sugar acceptor, and activity was determined by decrease in quercetin concentration in reaction mixture.
n.d.: Not determined.

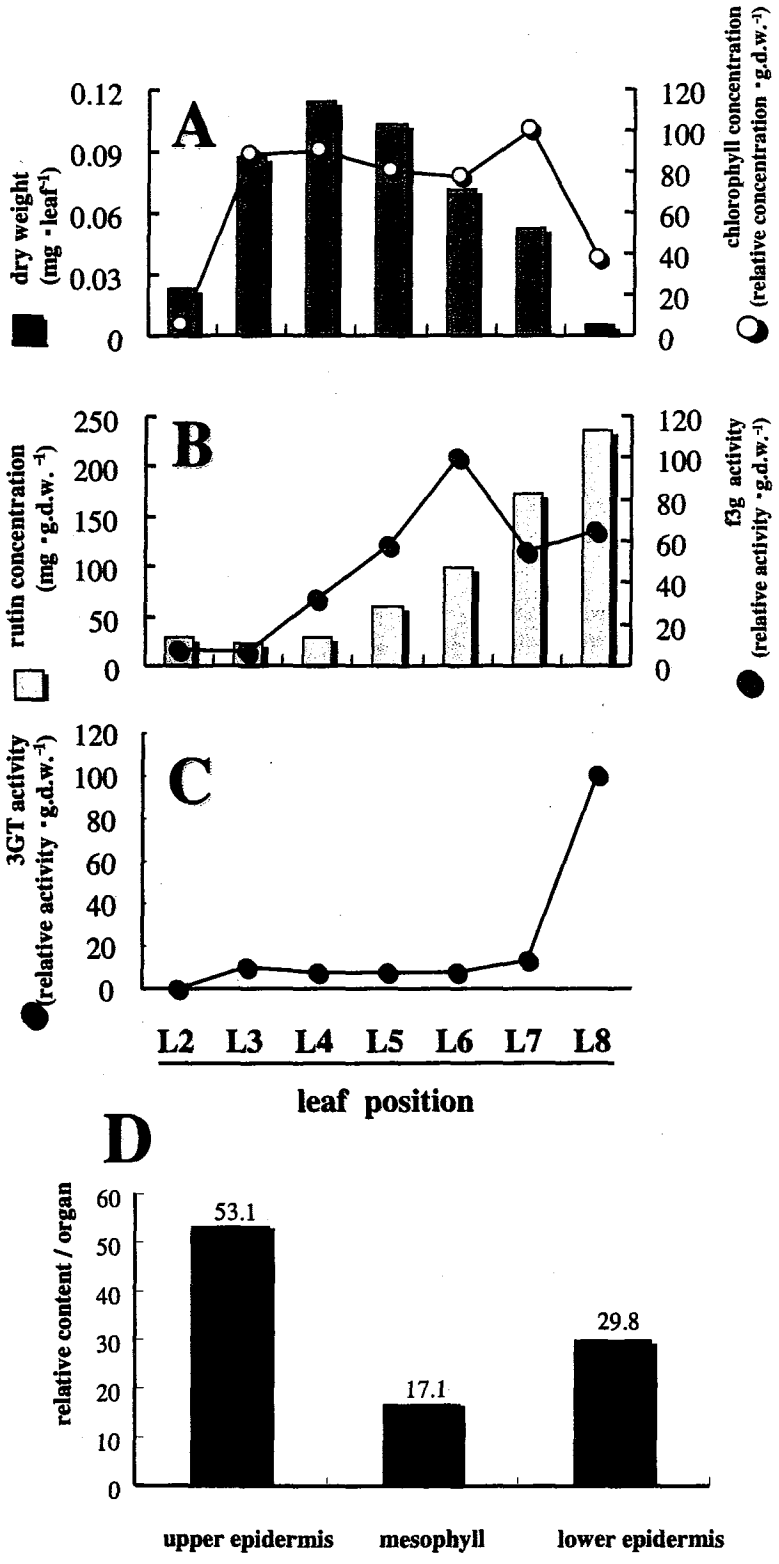



**Figure 2.** Measurement of rutin concentration, f3g activity, 3GT activity and organ distribution of rutin concentration of tartary buckwheat leaves.
Tartary buckwheat leaves from different leaf positions (L2; senescent leaf, L3 -L6; mature leaves, L7, L8; young expanding leaves) were harvested at 28 DAG from plants grown in an experimental field. Then, dry weight (A), chlorophyll concentration (L7 = 100)(A), rutin concentration (B), f3g activity (L6 = 100) (B), 3GT activity (L8 = 100) (C) and relative distribution of rutin concentration at L5 leaf (D) were measured. Data are means of two independent experiments.

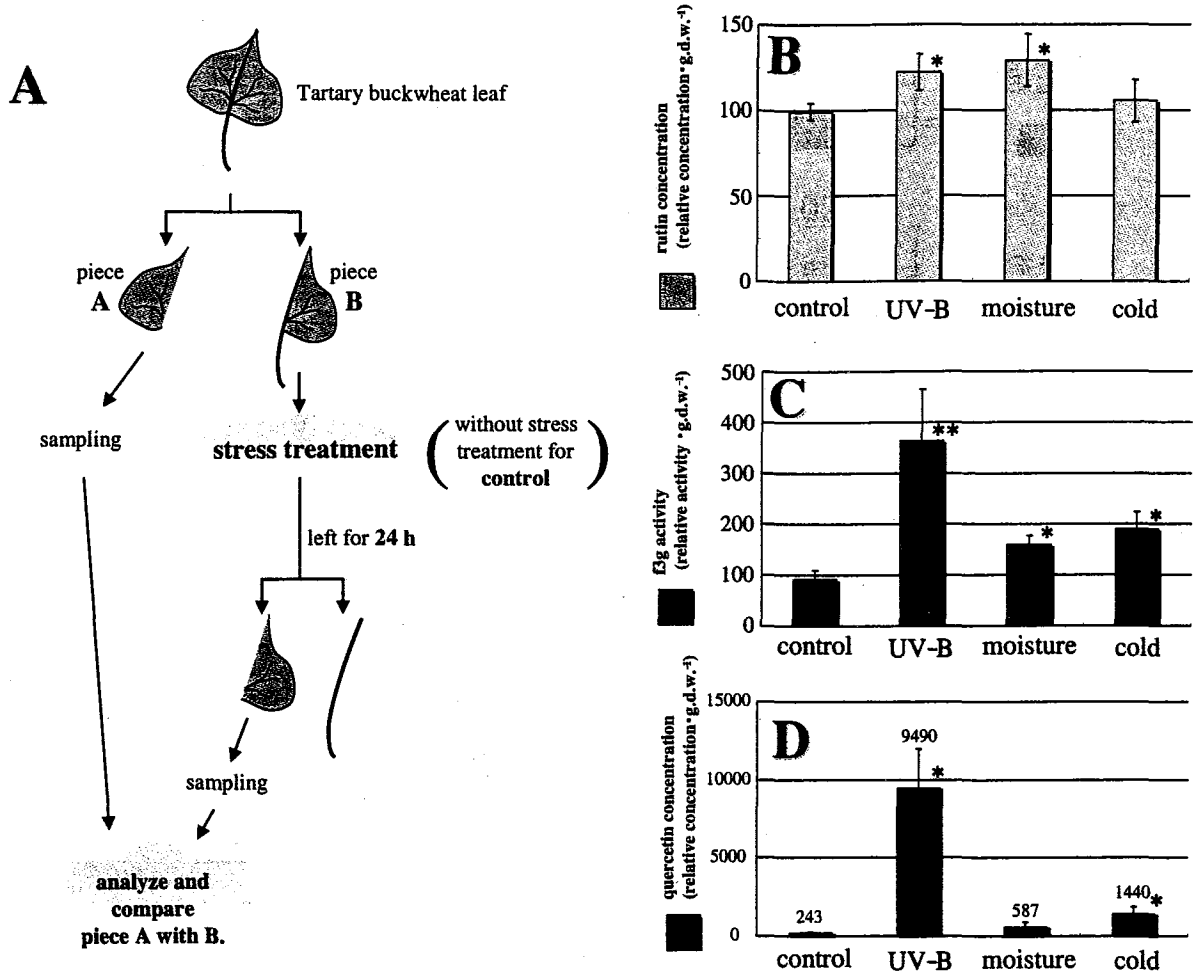



**Figure 3**. Effect of UV, moisture and cold stress applied to 28 days after germination 7th leaves of field-grown tartary buckwheat. (A) Flowchart of environmental stress treatments: UV-B radiation, desiccation treatment and cold treatment. As a control, an untreated leaf was used. Then, the treated leaf was left for 24 hour at 22℃ with adequate water. The rutin concentration and f3g activity were measured after removal of the vein, and the results of piece A and B were compared (value of piece A of each sample = 100). (B) Changes in rutin concentration. Data are means of five independent experiments. Bar indicates S.D. (C) Changes in f3g activity. Data are means of five independent experiments. Bar indicates S.D. (D) Changes in quercetin concentration. Data are means of three independent experiments. Bars indicate S.D.
*, **; Significant at the 5% level and 1% level by Student's *t*-test, compared with control.

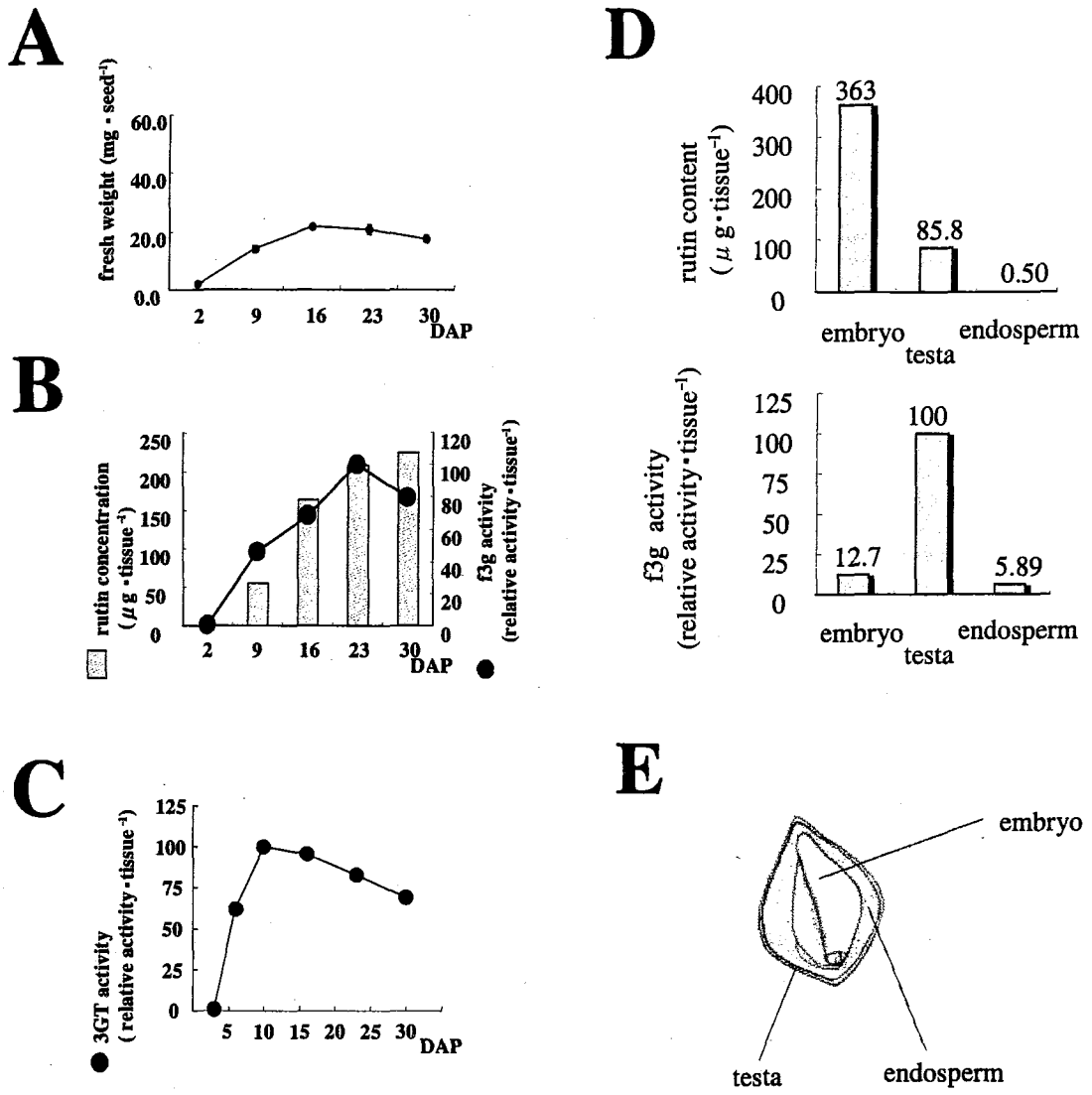



**Figure 4.** Measurement of rutin concentration, f3g activity, 3GT activity and organ distribution of rutin concentration and f3g activity of tartary buckwheat seeds during ripening.
A: Changes in fresh weight during ripening. Data are means of independent samples harvested from the same plant and SD values (n=3) are indicated. B: Changes in rutin concentration (bars) and f3g activity (lines) during ripening. A f3g activity was measured under standard assay conditions, and shown relative (%) to activity in 23 DAP (days after pollinating) seeds. Data are means of two independent samples harvested from the same individual. C: Changes in 3GT activity during ripening. 3GT activity was measured under standard assay conditions, and shown relative (%) to activity in 7 DAP seeds. Data are means of two independent samples harvested from the same plant. D: Organ distribution of rutin concentration and f3g activity in the fully ripe seeds of tartary buckwheat. Rutin concentration was measured using 10 seeds bulk of embryo or testa or endosperm, and f3g activity was measured under standard assay conditions. The values are the mean of two measurements. E: Illustration of a tartary buckwheat seed (husk removed).

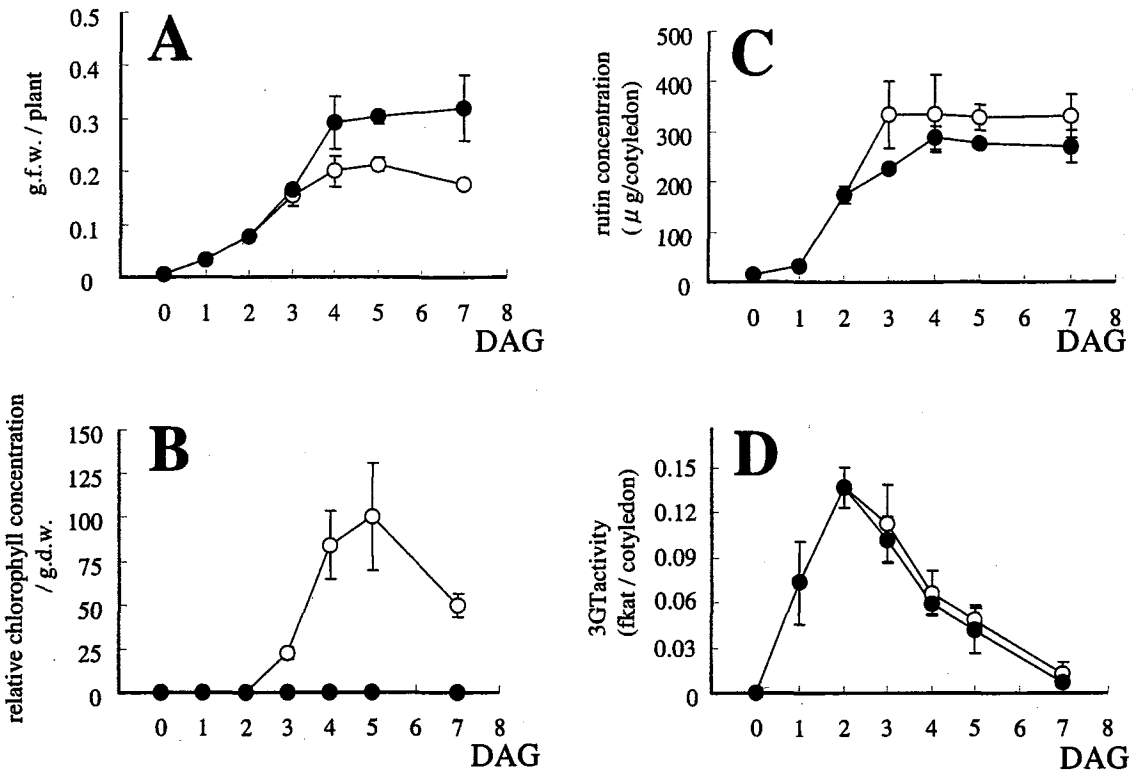



**Figure 5**. Measurement of rutin concentration, f3g activity, 3GT activity and organ distribution of rutin concentration of common buckwheat cotyledons grown with (○) and without light (●).
(A) plant growth; (B) changes in chlorophyll concentration relative (%) to that of 5DAG (days after germination) light-grown cotyledons; (C) rutin concentration in cotyledons; (D) 3GT activity in cotyledons. Data are means of three independent experiments. Bars indicate S.D

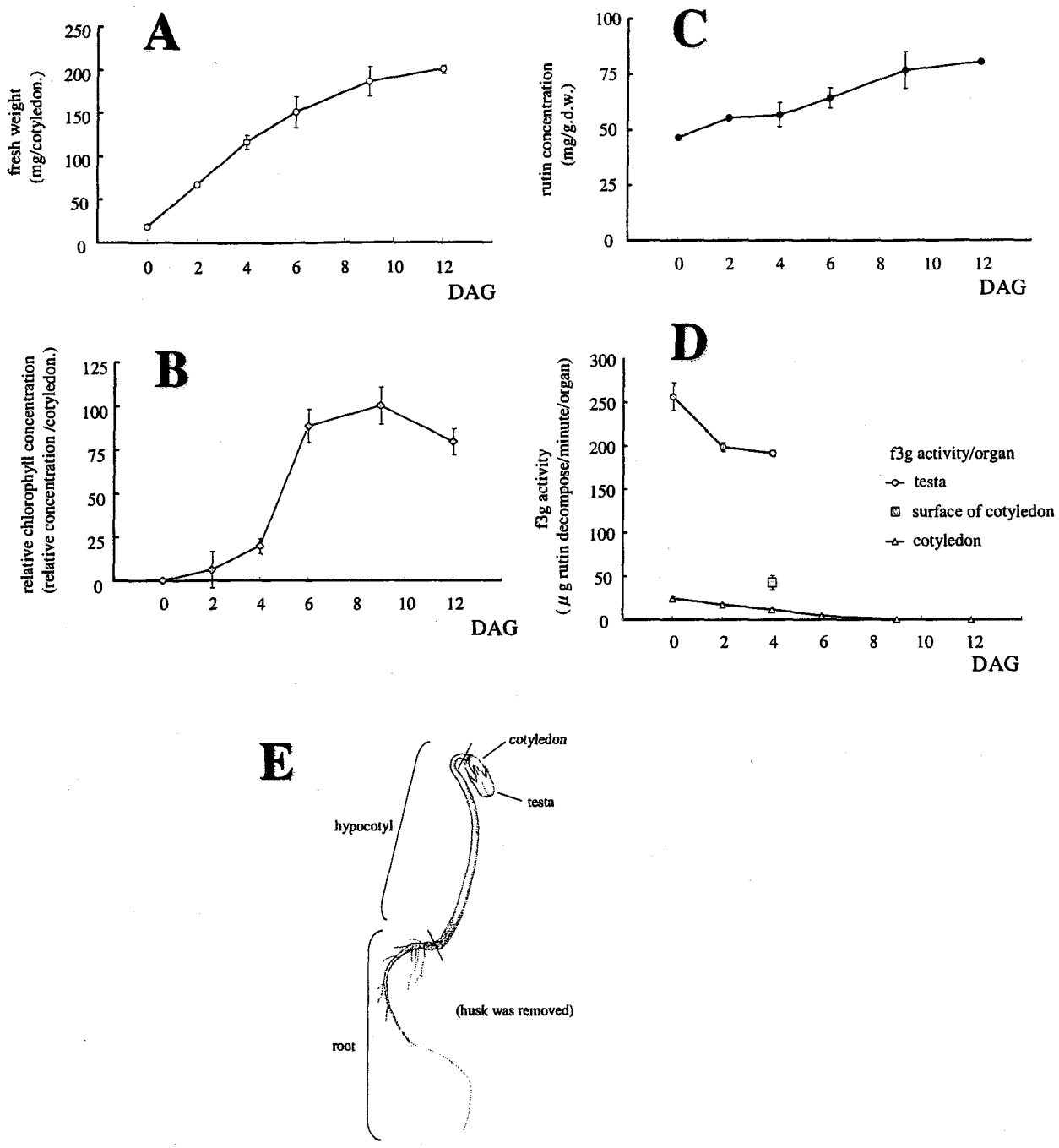



**Figure 6.** Changes in the f3g activity and rutin / quercetin concentration of tartary buckwheat cotyledons.

(A) plant growth; (B) changes in chlorophyll concentration relative (%) to that at 9 DAG (days after germination); (C) rutin concentration in the cotyledon . Rutin content was measured using HPLC; (D) f3g activity per organ; data are the mean of three independent experiments. Bars indicate S.D.; (E) Illustration of tartary buckwheat seedling at 4 DAG, with husk removed.

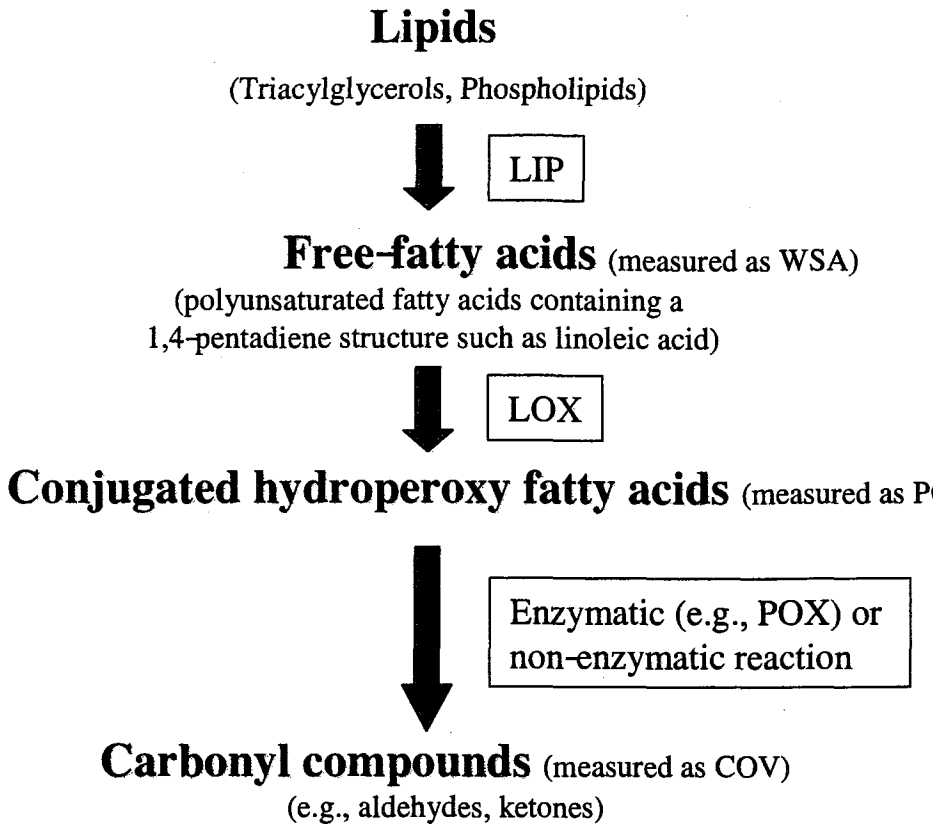



**Figure 7.** Model of lipid degradation proposed for rice bran. LIP: lipase, WSA: water-soluble acid, LOX: lipoxygenase, POV: peroxide value, POX: peroxidase, COV: carbonyl value

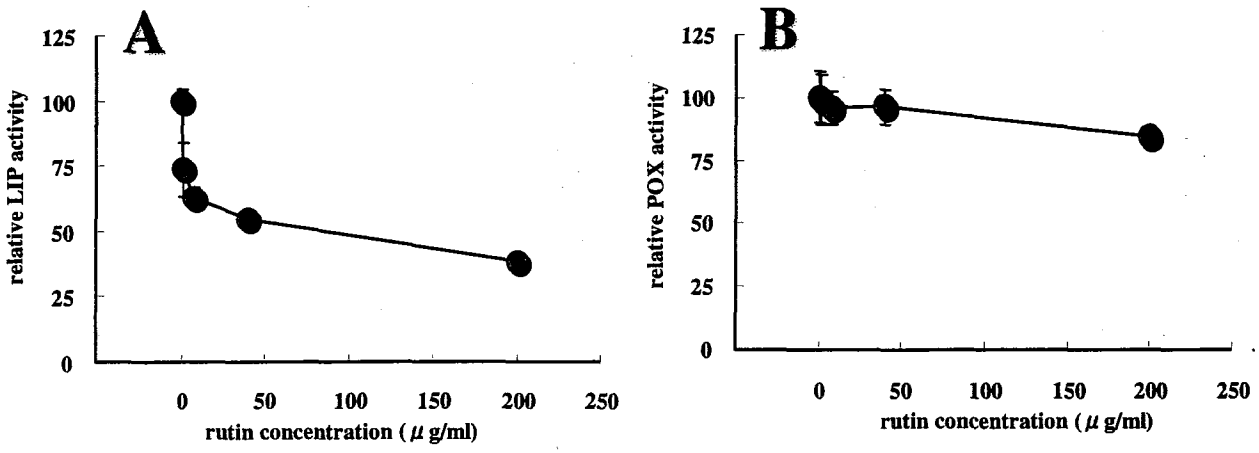



**Figure 8.** Inhibitory effect of rutin on *in vitro* common buckwheat LIP and POX activity
To investigate inhibitory effect of rutin against buckwheat LIP and POX activity, rutin was added in to the reaction mixture in which POX or LIP activity was measured. As substrate, *p*NPC12 (for LIP activity) or guaiacol (for POX activity) was used. Rutin was dissolved in EGME and added to the reaction mixture with final EGME concentration of 15%(v/v), and final rutin concentration of 0 to 200 μ g/ml. Reaction mixture contains LIP or POX activity corresponding to 66.6 mg buckwheat flour per 1 ml reaction mixture.
Relative LIP(A,C) and POX(B,D) activity expressed relative (%) to rutin-free enzyme activity. Data were means of three independent experiments. Bars indicate ±S.D.

**Table 3. Correlation matrix of values for measurements of Rutin, LIP, LOX, POX, pH, WSA, COV and POV from buckwheat flour stored at 5ºC or 20ºC.**

| | | Rutin | LIP | LOX 1 | LOX 2 | POX | Rutin | LIP | LOX 1 | LOX 2 | POX |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 5ºC | | | | | 20ºC | | | | |
| pH | 0 day | 0.45[a] | **-0.62**[b] | 0.09 | 0.12 | -0.10 | 0.45 | **-0.62** | 0.09 | 0.12 | -0.10 |
| | 4 day | 0.39 | **-0.55** | 0.16 | 0.23 | 0.04 | 0.01 | **-0.57** | 0.05 | 0.22 | 0.01 |
| | 10 day | 0.46 | **-0.77**[c] | 0.32 | 0.22 | -0.25 | 0.38 | **-0.61** | 0.39 | **0.54** | -0.04 |
| | 30 day | 0.34 | **-0.57** | 0.33 | 0.24 | 0.06 | 0.35 | **-0.66** | 0.45 | 0.43 | 0.12 |
| | 4-0 day | -0.14 | 0.14 | 0.16 | 0.24 | 0.32 | **-0.64** | 0.19 | -0.07 | 0.10 | 0.16 |
| | 10-4 day | 0.20 | -0.48 | 0.31 | 0.03 | -0.50 | **0.57** | -0.12 | 0.52 | 0.50 | -0.07 |
| | 30-10 day | -0.31 | 0.51 | -0.06 | -0.02 | **0.56** | 0.11 | -0.39 | 0.32 | -0.01 | 0.33 |
| WSA | 0 day | -0.50 | **0.64** | **-0.56** | -0.21 | 0.38 | -0.50 | **0.64** | **-0.56** | -0.21 | 0.38 |
| | 4 day | -0.45 | **0.71** | **-0.54** | -0.25 | 0.29 | **-0.55** | **0.66** | -0.45 | -0.15 | 0.37 |
| | 10 day | -0.51 | **0.63** | -0.51 | -0.26 | 0.34 | -0.35 | **0.60** | **-0.61** | -0.29 | 0.29 |
| | 30 day | **-0.55** | **0.63** | -0.52 | -0.24 | 0.33 | -0.50 | **0.61** | -0.48 | -0.25 | 0.30 |
| | 4-0 day | 0.23 | 0.01 | 0.18 | -0.03 | -0.29 | 0.03 | -0.15 | 0.46 | 0.24 | -0.16 |
| | 10-4 day | -0.37 | -0.03 | -0.10 | -0.11 | 0.27 | 0.24 | 0.16 | **-0.57** | -0.39 | -0.02 |
| | 30-10 day | -0.35 | 0.27 | -0.23 | -0.06 | 0.10 | -0.50 | 0.19 | 0.18 | 0.03 | 0.10 |
| POV | 0 day | 0.32 | 0.23 | 0.26 | 0.01 | 0.12 | 0.32 | 0.23 | 0.26 | 0.01 | 0.12 |
| | 4 day | 0.02 | 0.52 | -0.07 | -0.02 | -0.10 | -0.15 | **0.62** | -0.41 | -0.26 | 0.12 |
| | 10 day | 0.07 | -0.14 | -0.12 | -0.25 | **-0.60** | 0.45 | -0.14 | 0.39 | **0.61** | 0.17 |
| | 30 day | 0.12 | 0.08 | -0.40 | -0.19 | -0.37 | 0.20 | **0.62** | -0.24 | -0.32 | 0.17 |
| | 4-0 day | -0.30 | 0.22 | -0.31 | -0.03 | -0.20 | -0.42 | 0.42 | **-0.63** | -0.27 | 0.02 |
| | 10-4 day | 0.06 | **-0.60** | -0.07 | -0.25 | **-0.58** | 0.42 | **-0.61** | **0.60** | **0.62** | 0.01 |
| | 30-10 day | 0.04 | 0.31 | -0.31 | 0.14 | 0.45 | -0.07 | **0.67** | -0.45 | **-0.66** | 0.07 |
| COV | 0 day | 0.04 | -0.01 | -0.28 | -0.28 | -0.09 | 0.04 | -0.01 | -0.28 | -0.28 | -0.09 |
| | 4 day | -0.19 | 0.10 | 0.07 | -0.14 | -0.02 | -0.01 | 0.23 | -0.16 | 0.04 | -0.05 |
| | 10 day | 0.10 | -0.26 | 0.33 | 0.18 | -0.25 | 0.45 | **-0.74** | 0.42 | 0.45 | -0.26 |
| | 30 day | **-0.57** | 0.32 | -0.03 | 0.11 | 0.08 | 0.03 | 0.14 | -0.25 | -0.23 | -0.10 |
| | 4-0 day | -0.15 | 0.07 | 0.17 | 0.03 | 0.03 | -0.04 | 0.23 | 0.09 | 0.29 | 0.03 |
| | 10-4 day | 0.46 | -0.47 | 0.27 | 0.46 | -0.25 | 0.41 | **-0.82** | 0.49 | 0.37 | -0.19 |
| | 30-10 day | -0.48 | 0.44 | -0.29 | -0.07 | 0.26 | -0.38 | **0.75** | **-0.54** | **-0.55** | 0.16 |

a ( gray color ) : **not significant, b (black color): significant at 5% level, c (bold and under line ): significant at 1% level**

# 論文審査結果要旨

ソバは多くの国で栽培され，食用にされている。ソバにはフラボノイドの一種のルチンが含まれており，機能性食品として注目を受けている。しかし，ルチンを含むのは，穀物の中ではソバが唯一であり，ルチンの生合成や代謝，植物体におけるルチンの生理機能，さらに食品としてのソバ粉におけるルチンの役割など，不明な点が多々残されていた。

本研究では，ルチンの生合成に関わるフラボノール 3-*O*- グルコシルトランスフェラーゼ（3GT）と，分解に関わるフラボノール 3- グルコシダーゼ（f3g）の精製と動力学的解析，葉や種子におけるルチン含量と両酵素活性の成育に伴う変化，発芽幼植物におけるこれらの変化，環境ストレス条件下におけるこれらの変化などを詳細に検討し，ルチンの代謝と生理機能の一端を明らかにした。また，ソバ粉におけるルチンの機能を，特に脂質の酸化防止の観点から解析し，粉体にした後の品質劣化を防ぐ機能を持つことを明らかにした。

本研究から得られた結果は，次のようにまとめることができる。①動力学や基質の特異性から，f3g と 3GT はルチンの生合成と分解に関わる酵素であることを明らかにした。②ソバやダッタンソバを用いた生理的な解析から，ルチンは表皮細胞に特に集積することが判明し，ルチンは UV 遮蔽の機能を持つことを示唆できた。③発芽子葉や葉では，他の植物が集積するフラボノイドに比較して，ソバのルチン含量は非常に多く，食害に対する防御物質としての機能を持つ可能性を示唆できた。④特にダッタンソバでは，ルチンは乾燥・低温などの環境ストレスに対する防御システムを向上させる機能を持つことを示唆できた。⑤ソバ粉のリパーゼ活性をルチンが阻害することで，品質劣化を防ぐ機能を持つことを示唆できた。上記の成果を，国際学術雑誌に公表した。

以上，本研究の成果は，ソバの植物体においても，また食品としてのソバ粉においても機能の多くが不明であったルチンについて，数多くの新知見が得られたものである。モデル植物や主要作物で用いられている生化学，代謝生理学，植物科学，食品化学の技法をソバに導入し，育成に時間を要する材料であるのにもかかわらず，丹念に解析をすすめた優れた研究である。

本研究は，研究例の少ないソバに焦点をあて，機能性物質として脚光をあびているルチンの機能を，生理面と食品の面から明らかにした優れたものであり，審査員一同は，本論文提出者に対し，博士（農学）の学位を授与するに値するものと認定した。