Study of the effects of light on the quality of green asparagus spears cultivated by winter fusekomi forcing culture and basic gene expression analysis of flavonoid related genes

（グリーンアスパラガスの冬季伏せ込み促成栽培における補光処理が若茎の品質に及ぼす影響ならびにフラボノイド関連遺伝子の発現解析）

A Dissertation Submitted to

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CHAPTER 1

General Introduction

Asparagus (*Asparagus officinalis* L.) is a perennial plant belongs to *Liliaceae* family, originated from Mediterranean area and widely grown in the world because of its salt tolerance, hardy, heat tolerance, and also soil adaptivity. There are 3 types of asparagus spears namely, green, purple, and white. Green spears are the shoots and/or young spears commonly eaten as a fresh vegetable. Etiolated white spears is green asparagus produced by using two different culture methods and/or two blanching methods to block sunlight; the traditional soil-mound and the film cover method (Jishi et al.,2012). Purple asparagus has spears with purple or violet in color. There are at least 60 asparagus producing countries in the world with an estimated total area of 20 ha; China was considered as the first, following by Peru and Germany as the largest production area in the world (Benson et al., 2012). Asparagus is known to be rich in sugars, amino acids (such as asparagine), vitamins (such as A, B, C, E) and minerals (such as Ca, P, Fe, K, Zn) (Motoki et al., 2003). Asparagus contains some functional compounds beneficial for human health; green and purple asparagus contain anthocyanin and a certain amount of rutin; white asparagus contain protodioscin (saponin) (Chin et al., 2002; Maeda et al. 2005; Sun et al., 2007; Motoki., 2008; Maeda et al., 2010, 2012; Motoki et al., 2012).

Rutin is one of the major flavonoids that have been reported to show biological and pharmacological activities such as anti-inflammatory, anti-tumor, and anti-bacterial/viral properties along with potent radical-scavenging activity, as well as protective effects in protecting against capillary fragility and arteriosclerotic vascular changes (Calabro et al., 2005; Heellerstein et al., 1951; Guo et al., 2007).

Asparagus was first introduced to Japan in 1780s, however wasn't popular at that time. It became popular after World War II as sauce of canned white spears. In 1960s green spears became popular in Japan and spring harvest was the most popular method of harvesting spears, and the production area was spread up during these periods. Since 1980s, the mother-fern culture method was introduced and then used widely in Southwest area in Japan. Total annual production reached more than 50, 000 tons per year in 1990s (Maeda et al., 2012). Both the spring harvest and summer-autumn harvest using the mother-fern culture method are the most popular methods in Japan (Fig.2). On the other hand, in winter season, the domestic production of asparagus in Japan almost drops to zero; the price stays high substantially; and almost depends on the import from other asparagus production countries to fill the market needs (Fig.1).

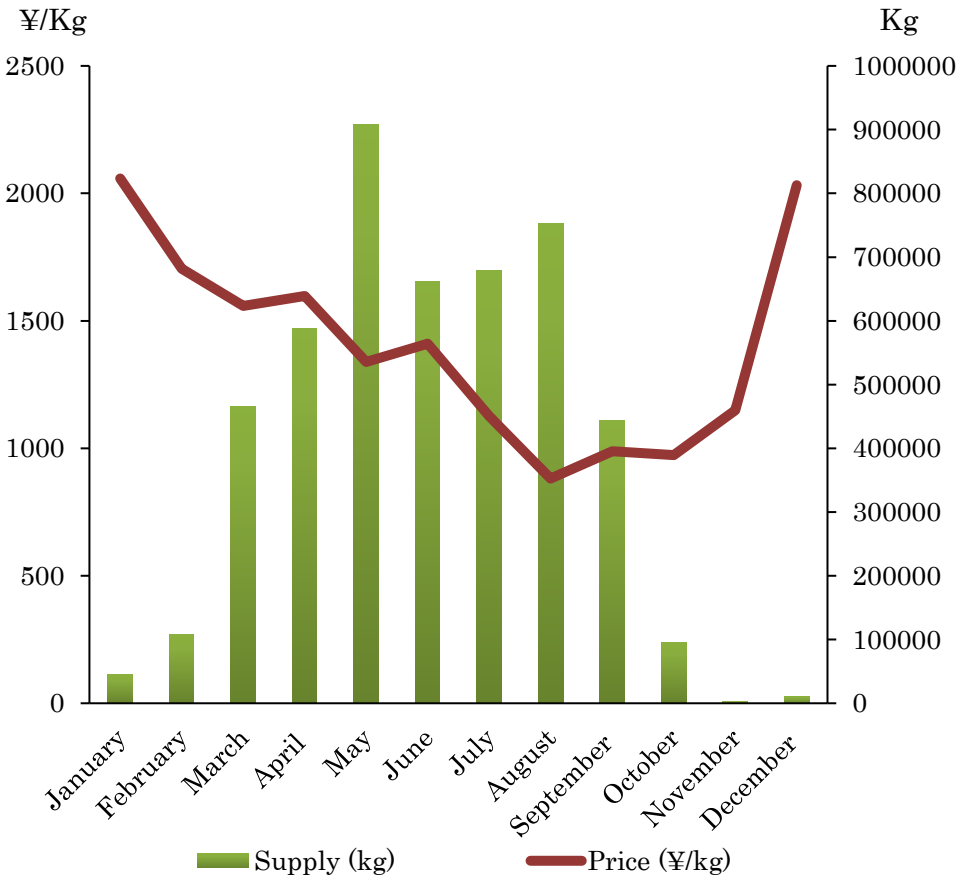

Fig.1 Statistic of asparagus domestic distribution in Japan

According to Tokyo Metropolitan Central Wholesale Market, 2016

Fig.2 Spring harvest (left) and Mother fern cultivation (right)

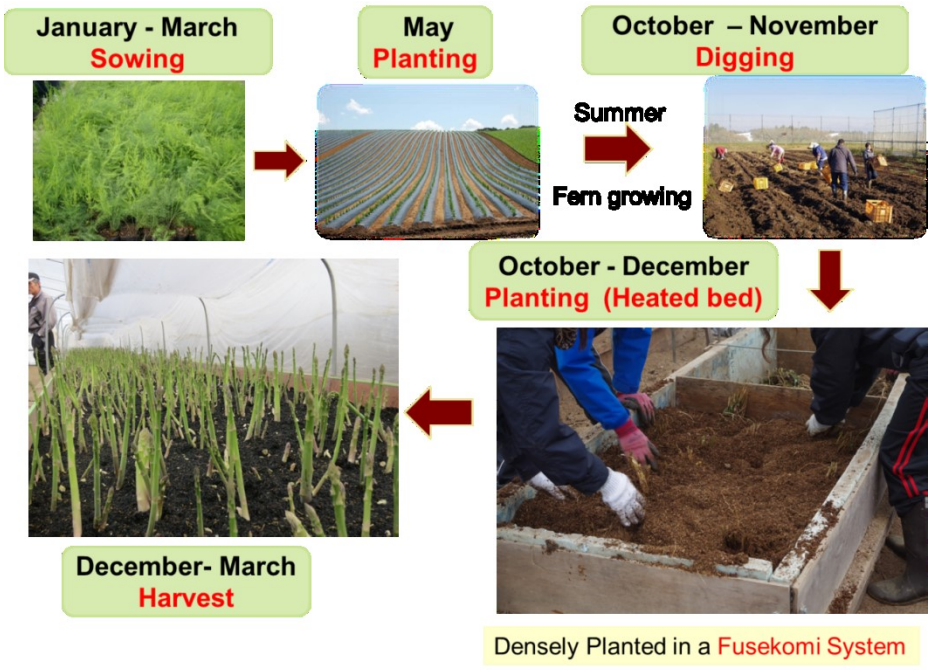

Fig.3 Winter 'Fusekomi' Forcing Culture

Fig.4 Spears in the Winter 'Fusekomi' Forcing Culture

Winter ‘Fusekomi’ forcing culture system has been conducted in Japan to make asparagus production possible during this season (Koizumi et al., 2003) by heating one-year-old rootstocks in a ‘Fusekomi’ forcing system.

In January, the seeds are sown and grown in a green house. On early May, the seedlings are transplanted into an open field. After the yellowing of the fern in Autumn (October-November), the rootstocks then are dug up from the field and planted densely in the ‘Fusekomi’ forcing system. In the ‘Fusekomi’ forcing system, heating wires were already set under the bed to warm the rootstocks, and the rootstocks were covered by the soil from the upper sides. After all this process, finally even in the winter, asparagus could be produce (Fig.3). However, in this method, due to the low sunlight, especially in the production area which has much snow and short sunshine, spears are mostly growing under small amount of light. These conditions cause some problems, such as, the color of the spears is pale, and rutin contents are relatively lower compare to those growing in the spring (Fig.4). The same problem also has been found in the mother-fern culture method. In the mother fern culture, spear that are grown and harvested mostly in the shadows of mother ferns results in problem such as low quality of spears, in particular, spear are paler than those that harvested in the spring and rutin content also lower compare to those harvested in spring. On the other words, these problems are

affected by low levels of light and/or UV radiation. Therefore by improved light conditions or light environment, rutin content were increased and spear color also becomes better (Maeda et al., 2010). According to our preliminary study, rutin content tends to be increase when the asparagus is cultivated under supplementary lamp (fluorescent lamp) and also the color of the spear becomes better than those in the control plot or no supplementary lamps (data not shown). These results suggested that by improving the light condition during winter-forcing culture, the quality (rutin content and spears color) of spears also could be improved. It has been reported that flavonoids and phenolic shows selective UV-B filter which protect plant tissue against harmful rays, and the response also differs according to the light quality (UV), species and environment (Rozema, et al., 2002; Kreft et al., 2002). Study in Arabidopsis (*Arabidopsis thaliana*) shows that flavonoind biosynthetic genes are effectively express by UVB light with blue light induction than in dark condition (Kubasek et al., 1992). Study in buckwheat gene expression showed that, the latest gene on flavonoid pathway; F3'H and FLS, were substantially upregulated at 2, 4, and 6 days in light/dark-treated than in dark-treated one (Li et al., 2010, 2012). These studies demonstrated that rutin and/or flavonoids are fluctuates with light intensity or light environment. However, only few study about rutin and its relationship with light in asparagus. Studies about enzymes

involved in flavonoids biosynthesis pathways on asparagus have not yet been identified and there is limited information about its gene expression in asparagus.

Therefore, the objective of this study was to investigate the effect of supplemental lighting on the yield, rutin content, and spears color by using different irradiation time, different quantity, and quality of light on the winter ‘Fusekomi’ forcing culture and also to clarify the mechanism of rutin increases from molecular biological aspects.

CHAPTER 2

Effect of Supplemental Light on the Quality of Green Asparagus Spears in Winter 'Fusekomi' Forcing Culture

INTRODUCTION

Green asparagus contain rutin (Chin et al., 2002; Sun et al., 2007; Maeda et al., 2010; 2012), a compound with antioxidant properties, good for human health (Jang et al., 2004; Rodriguez et al., 2005). Fusekomi forcing culture system has been conducted to produce asparagus in the winter (Koizumi et al., 2003). However, in these methods, spears are mostly growing under small amount of light because of much of snow and low sunlight in the production area. It causes some problems such as, the color of the spear is pale, and rutin content is lower compare to those harvested in spring. Fusekomi forcing culture is conducted in a relatively small space with dense planting (planting density is more than twenty times, comparing to the open field cultivation), therefore 'environment control' is easier to conduct by introducing supplemental lighting into this system. So the aim of this study was to obtain basic knowledge to improve the quality (color and rutin contents) of asparagus spear in order to produce high value of the spear during winter season by using different irradiation time (2012) and different quantity of fluorescent light (2013) on the winter 'fusekomi' forcing culture.

MATERIALS AND METHODS

Preparation for experiment by using winter 'fusekomi' forcing culture

Trial on 2012 was conducted to evaluate the effects of different irradiation time on asparagus spear and trial on 2013 was conducted separately in purpose to investigated the effect of different quantity of fluorescent light. Preparation for the experiments was conducted as follows; one-year-old rootstocks of 'UC157' were dug up from an open field of Iwate Agricultural Research Center on December 6, 2011, or November 22, 2012. The rootstocks had been planted in the heated cultivating beds ('fusekomi' system) set in a greenhouse at the trial field of Hirosaki University on December 16 to 19, 2011 or November 30 and December 3 to 4, 2012. Thirty to forty rootstocks were planted for one plot (80 cm x 120 cm x 45 cm), and were covered with rice hull compost. All the test plots were covered with the plastic tunnels and the house was covered with a shading net (only on 2013 trial). The minimum air temperature was kept higher than 10°C using an oil heater and soil temperature was kept around at 18°C using heating wires.

Experimental treatments by using winter 'fusekomi' forcing culture

In 2012, three trial plots with 2 repetitions were prepared as follows: Control; with no supplemental light, FL1-16; 1 supplemental lighting for 16 h a day (FHF 32EX-N-H,

Panasonic Co., Japan), and FL1-24; continuous (24 hours a day) lighting with 1 white fluorescence lamp. All the test plots were covered with a light reflecting material Tyvek sheet (DuPont-Asahi Flash Spun Products Co., Japan) during the night (Fig. 5). The trial where conducted from January 16 to March 5, 2012. In 2013, three trial plots with 2 repetitions were prepared as follows: Control; with no supplemental light, FL1- 24; continuous (24 h a day) lighting with 1 white fluorescence lamp (FHF 32EX-N-H, Panasonic Co., Japan), and FL2-24; continuous (24 h a day) lighting with 2 white fluorescence lamps. All the test plots were covered with a light reflecting material Tyvek sheet (DuPont-Asahi Flash Spun Products Co., Japan) during the night (Fig. 5). The trial was conducted from January 15 to February 25, 2013.

Light intensity and air temperature measurement

Light intensity at night and daytime, and air temperature of the plots were measured during the harvest period. Light intensity was measured under different weather, with 24 cm height from the compost level by using photo quantum meter (Kaito memory sensor MES-136 light logger, Yokohama, Japan). Air temperatures were recorded by using thermo recorder (T&D wireless thermo recorder RTR-52A, Nagano, Japan).

Spear weight observation and spear color analysis

Yields observation were difficult to conducted in this study because of the limited plots, so we used the average of spear weight per plot as the parameter to observed the effect of irradiation time on yield in this experiment, consider spear weight also are the important factor for yields. Spears were harvested at 27 cm and cut to a length of 24 cm for weight observation. The weight of spears in each plot was measured every day during the harvest period. Spear color was investigated by measuring L* (lightness), a* (red-green), and b* (yellow-blue) values in the bottom section (34 cm from the cut) of M (middle; 13-18 g) to L (large; 18-23 g) sized spears in each plot. The values were obtained using an NF333 (Nippon Densyoku Industry Co., Japan) color difference meter and the reconstruction of color and/or the average of L*a*b* value were obtain by using the software 'Irodashimeijin for windows'.

Determination of rutin and sugar contents

For component analysis, rutin and sugar content observation were determined. Three to four of M (middle; 13-18 g) to L (large; 18-23 g) sized spears in each plot were cut into 3 sections of equal length (8 cm of each section), immediately frozen, and then lyophilized. Because rutin have been reported to be highest in the spearheads or top section (8 cm from the spears tip) while low in the bottom part. So, only the spearheads

was specifically examined to investigate rutin contents (Chin et al., 2002; Maeda et al., 2008). The bottom section was used for sugar analysis (fructose, glucose, sucrose). Rutin were extracted from 20 mg of freeze-dried powder of M to L size spears (11 to 20 g) from each experimental plot by using 1 ml of 80% methanol. The extraction was conducted for three hours at room temperature. Sample solutions were then centrifuged (10,000 rpm, 10 min) and filtered. Rutin content was determined by high performance liquid chromatography (HPLC) as described by Maeda et al. (2012). HPLC analysis was conducted using a Waters Sunfire C18 (4.6 x 250 mm) column. The mobile phases were consisted of 0.1% trifluoroacetic acid (A) and acetonitrile (B). Analysis was performed by running each sample for 30 min at a column temperature of 40°C, using a linear gradient system at flow rate of 1.0 ml min^{-1} and each run was monitored at a wavelength of 354 nm UV detector. The gradient condition was 0 min, 84% solvent A and 16% solvent B; 20 min, 60% solvent A and 40% solvent B; 30 min, 40% solvent A and 60% solvent B, and the post running time was 10 min.

Sugars were extracted from 20 mg of the freeze-dried powder with 1 ml of 70% ethanol for 1 h at room temperature. The extracted solution was centrifuged, and the supernatant was used for sugar analysis. Sugar content was determined by using HPLC system equipped with a Shodex Asahipak NH2P-50 4E (4.6 x 250 mm) column. The mobile

phases consisted of 78% acetonitrile. Analysis was performed by running each sample for 20 min at a column temperature of 35°C, with a flow rate of 1.0 ml min^{-1} each run was monitored by evaporative light scattering detector (Model 300S ELSD, M&S Instruments Inc., Japan).

RESULTS

Light intensity and air temperature inside the tunnel on each test plot

In order to examine the effect of supplemental lighting to the environment inside the tunnel, light intensity at night and daytime, air temperature of the plots were measured during the harvest period. The average of light intensity at night and daytime on control, FL1-24, and FL2-24 were as follows; 0, 59, 143 μmol m^{-2} s^{-1} and 68, 103, 163 μmol m^{-2} s^{-1} (Table 1). The average of air temperature inside the tunnel on control and FL2-24 were 11.9°C and 17.2°C, respectively. In addition, the average of the air temperatures on the aisle between examination beds was 10.6°C.

Effect of irradiation time on rutin content (2012)

On 2012, during the harvest period rutin content of control shows 2.14 mg g dry $weight^{-1}$ (DW) , followed by FL1-16 and FL1-24 with 2.34 and 2.56 mg g^{-1} DW of rutin average (Fig. 6). Rutin content on the FL1-16 and FL1-24 plots were higher than in the control plot significantly (Tukey-Kramer test, $P<0.05$, $n=18$).

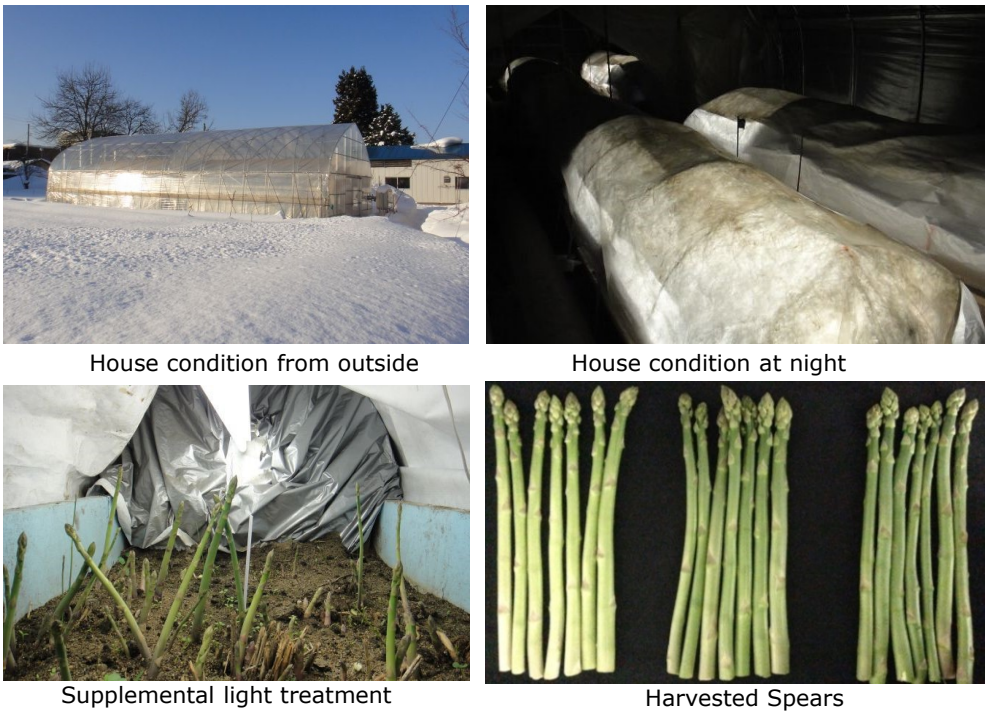

Fig. 5 Photographs of the experiments conducted by winter 'fusekomi' forcing culture

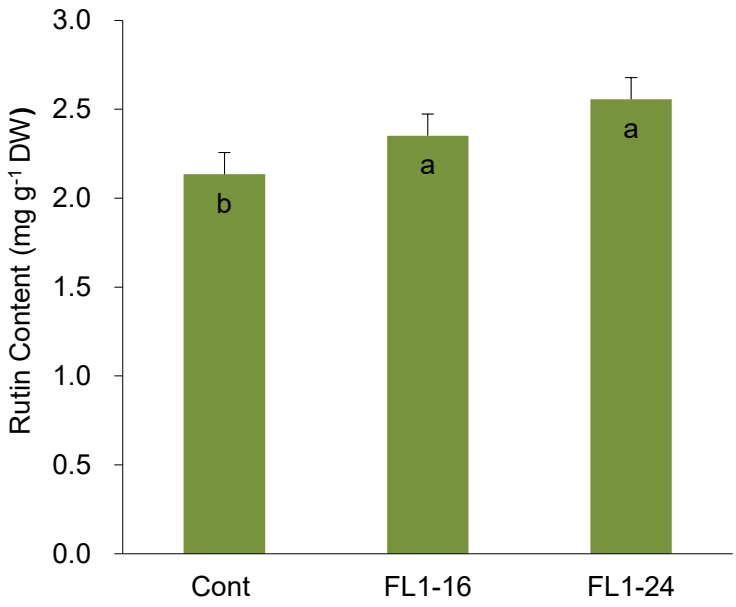

Fig. 6 Effects of irradiation period of supplemental lighting on the rutin contents in the asparagus spears harvested by winter 'fusekomi' forcing cultivation on 2012. Bars indicate SE. Different letters indicate significant differences (Tukey-Kramer test, $P<0.05$, $n=18$)

Effect of different quantity of fluorescent light on rutin content (2013)

We confirmed that rutin content was increased in the both supplemental lighting plots, FL1-24 and FL2-24 (Fig. 7). Control plot showed 1.66 mg g^{-1} DW of rutin average. FL1-24 and FL2-24 were containing; 2.45 and 2.61 mg g^{-1} DW of rutin, respectively. FL1-24 and FL2-24 showed the highest rutin content, and there was a significant difference against control (Tukey-Kramer test, $P<0.05$, $n=18$)

Effect of different quantity of fluorescent light on spear color (2013)

The result of color difference, L* value of the spears harvested at control, FL1-24, and FL2-24 plots were 59.35, 53.54, and 49.54, respectively (Fig. 8). Compare to the control plots, both supplemental lighting plots showed low L* value, which meant that the spear color became deeper. Significant differences were observed among the plots by Tukey's test $P<0.05$ on L* value. L* value of the spears harvested at supplemental lighting plot were significantly low compare to the control plot. Also, within the supplemental lighting plots, L* value of the spears harvested at the plot of FL2-24 was lower than that of the FL1-24 plot, significantly (Tukey-Kramer test, $P<0.05$, $n=41$-63). Both a* and b* value of the spears harvested at control, FL1-24, and FL2-24 were measured. The values of a* and b* on control, FL1-24, and FL2-24 were as follows: -7.64, -13.36 and -12.70; 20.95, 31.04 and 28.13, respectively. a* values on FL1-24 and FL2-24 shows a

significant difference compare to the control, same trend also have been observed in b* values, both supplemental lighting plot has a significant difference compare to the control (Tukey-Kramer test, $P<0.05$, $n=41\text{-}63$).

Effect of different quantity of fluorescent light on sugar content (2013)

Sugar contents were determined during the harvest periods (Fig. 9). The average of sugar contents was stable during the harvest period and there was no significant differences observe among the experimental plot. These results indicated that there was no negative impact of supplemental lighting on sugar contents.

Effect of different quantity of fluorescent light on spear weight (2013)

The yields observation were conducted every day during harvest period, we measured the weight of spear and took average only from the spear from 8 g to 23 g. The average of spear weight of control plot, FL1-24 and FL2-24 plot were 16.7 g, 15.8 g, and 14.8 g, respectively (Fig. 10). No significant difference was observed between control and FL1-24, however, there was a significant difference between control and FL2-24. Compare to the average of spear weight on control, FL2-24 spear's become a bit lighter (Tukey-Kramer test, $P<0.05$, $n=400\text{-}500$).

Table 1 The average of light intensity and air temperature during harvest period.

	Light Intensity (μmol m^{-2} s^{-1})				Average Air Temperature (°C)[x]
	Daytime[z]		Night[y]		
	Averagae	Range	Average	Range	
Control	68	21−136	0	0	11.9
FL1-24	103	44−171	59	40−78	–[w]
FL2-24	163	77−243	143	112−176	17.2

[z] Measured by using photo quantum meter under different weather at around noon during harvest period.

[y] Measured by using photo quantum meter after the sunset (completely dark period).

[x] Recorded by using thermo recorder every 20 min during the harvest period.

[w] Not recorded.

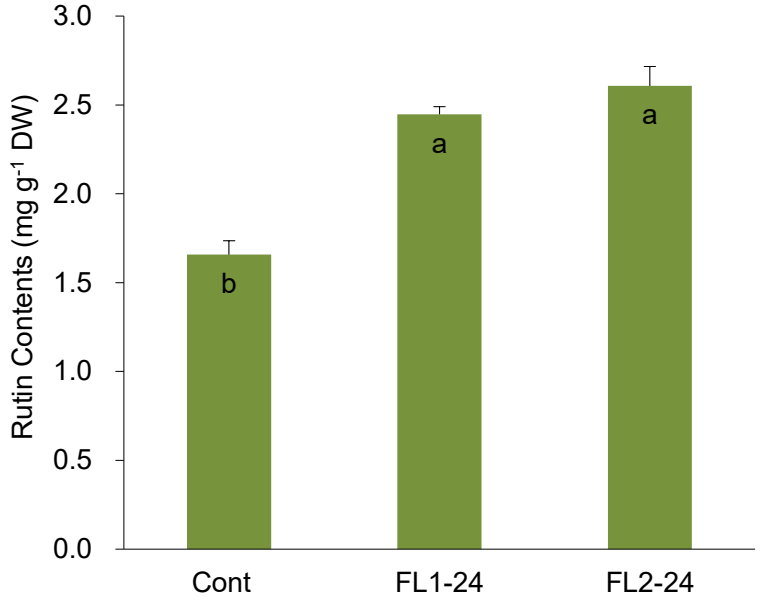

Fig. 7 Effects of supplemental lighting numbers on the rutin contents in the asparagus spears harvested by winter 'fusekomi' forcing cultivation on 2013. Bars indicate SE. Different letters indicate significant differences (Tukey-Kramer test, $P<0.05$, $n=18$)

DISCUSSION

The effects of supplemental lighting on spear color were observed. L* value defines lightness if the values near hundred, and in contrast, if the value are low and/or near zero, it defines dark in color. We found that L* value of the spear harvested at supplemental lighting plots, were significantly lower, compare to the control, even within the supplemental lighting plots, L* value of spears harvested at the plot of FL2-24 showed significantly lower than that of the FL1-24 plot. a* and b* value also on both supplemental lighting shows a significant difference against control. These results suggested that the color of the spear became deeper than the control plots in association with numbers of the fluorescent lamps, and it lead to be better color, distinguishable with naked eyes (Fig. 11).

Sugar composition in the spears showed similar results to other former studies, consisting 50-54% of fructose, 42-44% of glucose and 4-5% sucrose (Matsubara, 1981; Shou et al., 2007; Brueckner et al., 2010), and there was no significant difference among experimental plot observed. So, it is clear that there was no negative impact of supplemental lighting on sugar content.

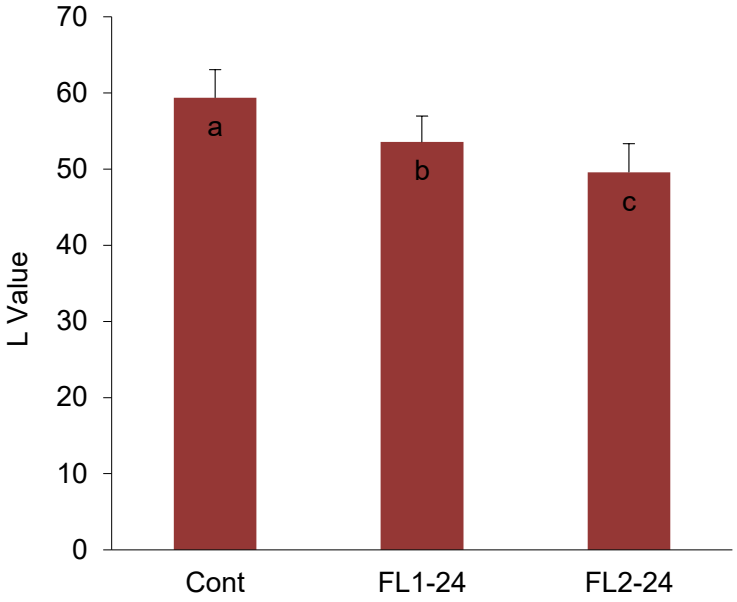

Fig. 8 Effects of supplemental lighting on color of the spears (L* value, measured by a color difference meter). Bars indicated SE. Different letters indicated significant differences (Tukey-Kramer test, $P<0.05$, $n=41$-63).

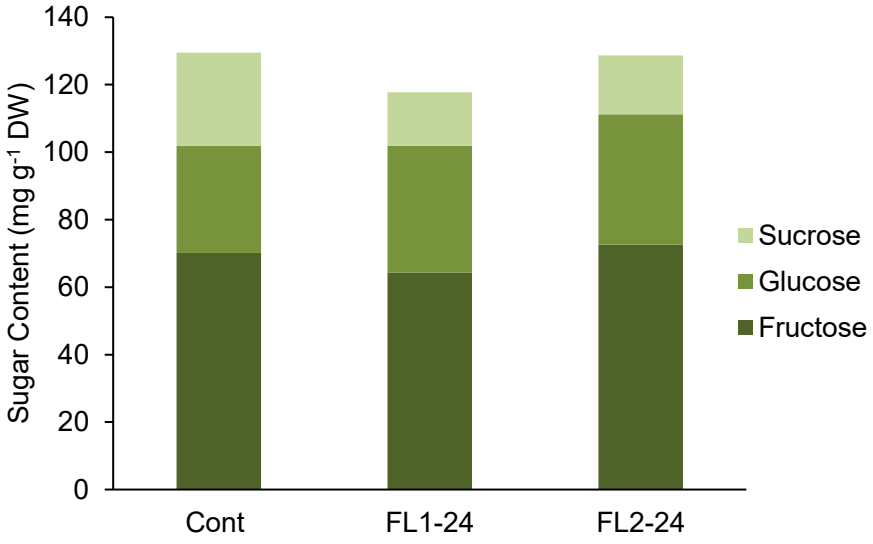

Fig. 9 Effects of supplemental lighting on the sugar contents in the asparagus spears harvested by winter 'fusekomi' forcing cultivation. No significant difference among examination plots (Tukey-Kramer test, $n=18$).

The investigation of rutin content by using different irradiation time (2012) and different quantity of fluorescent light (2013) shows that rutin were increased significantly by the presence of fluorescence lamps. On 2012, rutin content on FL1-24 treatment were significantly increased against control and even 16 h treatment tended to be increase. The same trends also were observed on 2013 trial. Rutin content on FL1-24 and FL2-24 h treatment were significantly increased against control plot. The relations between light and rutin (flavonoids) have been studied well and reported that the rutin played a role as protector against ultraviolet (UV) in living plants (Kubasek et al., 1992; 1998; Wade et al., 2001; Rozema et al., 2002; Ebizawa et al., 2008). In the mother-fern culture during summer to autumn harvest, by improving the light environment rutin content were significantly increased (Maeda et al., 2010). Study in buckwheat showed that rutin getting higher according to the light such as UV-B specifically (Lee et al., 2001; Kreft et al., 2002; Li et al., 2010; 2012). The trial in arabidopsis (*Arabidopsis thaliana*) also shows that flavonoids biosynthetic genes were effectively expressed by the induction of UV-B light and blue light, though none in dark condition (Kubasek et al., 1992; Fuglevand et al., 1996). These studies demonstrated and/or indicated that rutin content tends to fluctuate according to the light environment.

The results of rutin content on this study and the correlation with light intensity of each plot, FL1-16, FL1 and FL2 for 24 h plots shows that spears grown under high light intensity than in control; rutin content getting higher due to the presence of supplemental lighting and the length of exposure time (Fig. 8). Therefore this result suggested that rutin biosynthesis in asparagus spears was enhanced by supplemental lighting because rutin content in control plot during the harvest period stayed lower compared to the supplemental lighting plots, which has higher amount of rutin.

We found that there was no negative impact on spear weight; however the spear seems to be a bit lighter on the FL (florescent lamp) plot. It is thought that during the harvest period, except the control plots, spears grown in the FL2-24 were irradiated almost 6 week under 2 lamps for 24 h and it affected directly to the air temperatures inside the tunnels. The air temperatures were about 6°C higher in the continuous supplemental lighting compare to the control. So these, might be the factor of dryness of the compost during examination, therefore affect the spears growth and also the weight of spear.

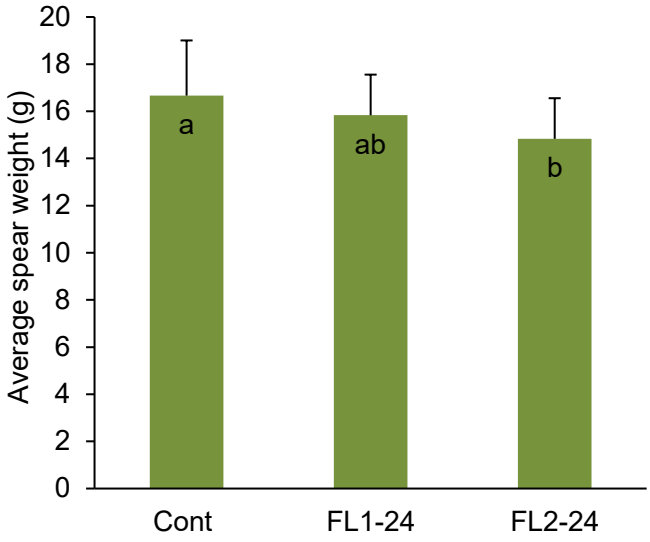

Fig. 10 Effects of supplemental lighting on the average spear weight of the asparagus spears harvested by winter 'fusekomi' forcing cultivation. Bars indicated SE. Different letters mean significant differences (Tukey-Kramer test, $P<0.05$, n=400-500).

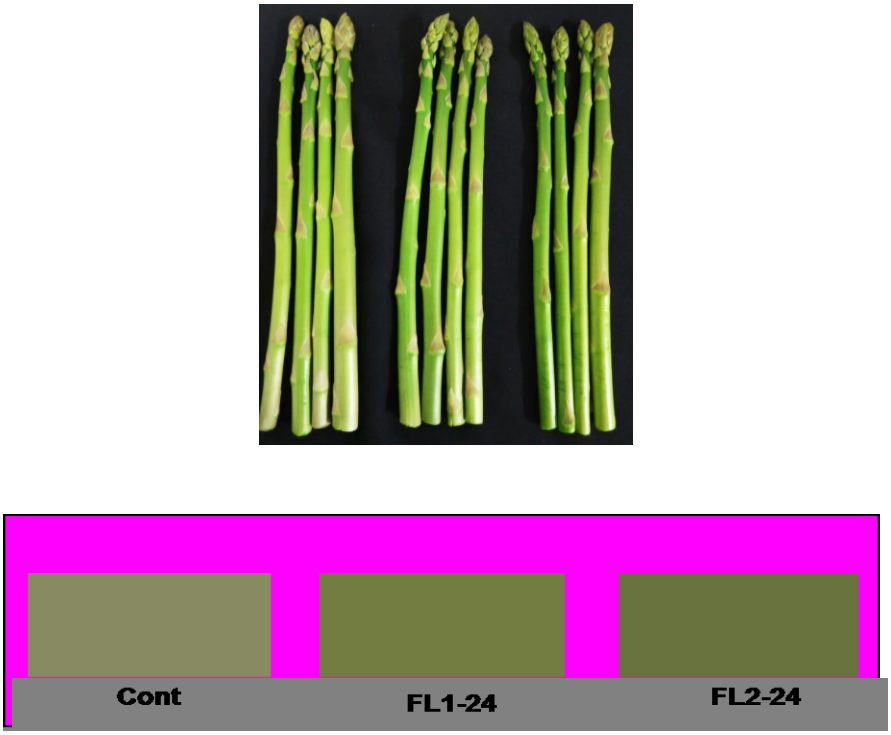

Fig. 11 Differences in spear color harvested on 2013 trial and the reconstructed color of L*a*b* average values (n =41-63) by using the software 'Irodashimeijin for windows'.

Further studies are needed to clarify the effect of supplemental lighting on the yield and/or spear weight, in order to find the best condition, and also to see the limitation of the light control to the plant. At the same time, unlike others plant such as, buckwheat, and arabidopsis, the enzymes involved in flavonoids biosynthesis pathways on asparagus have not been identified yet (Kubasek et al., 1992; Li et al., 2010; 2012). Particular studies about mechanism of the rutin increase by supplemental lighting in asparagus from molecular biology aspect are needed. We want to find the key role of the flavonoids gene in asparagus.

As the conclusion, the results of this study showed that the method was effective to improve the quality of asparagus spears, especially for the area that has low sunlight in winter season. And beside it is easy to conduct and could help to improve spear quality, it also could keep temperature inside the tunnel warm.

CHAPTER 3

Effects of different Quality and Quantity of Supplemental Lighting on the Quality of Green Asparagus Cultivated by Winter 'Fusekomi' Forcing Culture

INTRODUCTION

Plants synthesize a wide variety of flavonoid compounds with important functions in disease resistance, protection from UV radiation, and coloration (Harborne and Grayer, 1993; Mol et al., 1998; Bieza and Lois, 2001; Glories, 1988; Mateus et al., 2002). Higher plants are capable of sensing and responding to environmental light signals, discriminating among wavelength, intensity, and duration (Gao et al, 1994). At least three classes of photomorphogenic system are available for light perception as defined by the wavelengths absorbed by the photoreceptor: (a) phytochromes (responding primarily in the red and far red) (Furuya, 1993), (b) blue receptors or cytochrome (Ahmed and Cashmore, 1993; Kaufman, 1993), and (c) UV-B receptors (Gao et al., 1994). Different light quality such as, UV-A, UV-B, and blue light have been reported to enhance rutin (Jenkins et al., 1997; Lee et al., 2001). As described in the previous chapter; different number of supplemental lighting, light intensity (light quantity) and different irradiation time affects the amount of rutin. In this chapter, in

order to confirm the effects of light on the quality of asparagus spear and also to find the best conditions to produce high quality asparagus during winter season. So, tests by using different quantity of fluorescent light and different quality of light on the winter 'fusekomi' forcing culture were conducted.

MATERIALS AND METHODS

Experimental treatment by using winter 'fusekomi' forcing culture on 2014

Preparation for the experiments on 2014 was conducted as follows; one-year-old rootstocks of 'UC157' were dug up from an open field in Iwate Agricultural Research Center on November 19, 2013 and had been planted in the heated cultivating beds (length 1.2 m x width 0.8 m x height 0.45 m for each plot) set in a greenhouse at the University field on December, 6, 2013. Thirty to forty rootstocks were planted for one plot (80 cm x 120 cm x 45 cm), and were covered with rice hull compost. All the test plots were covered with the plastic tunnels and the house was covered with shading net. Three trial plots with 2 repetitions were prepared as follows: Control; with no supplemental light, FL1-24; continuous (24 hours a day) lighting with 1 white fluorescence lamp (FHF 32EX-N- H, Panasonic Co., Japan), and FL3-24; continuous (24 hours a day) lighting with 3 white fluorescence lamps. All the test plots were covered with a light reflecting material Tyvek sheet (DuPont-Asahi Flash Spun Products Co., Japan) during the night (Fig. 5). The minimum air temperature was kept more than 10°C using an oil heater and soil temperature was kept around 18°C using the heating wires. The trial was conducted from January 14 to February 24, 2014 (harvest period-6 weeks).

Experimental treatments by using winter 'fusekomi' forcing culture on 2015

Preparation for the experiments on 2015 was conducted as follows; one-year-old rootstocks of 'UC157' were dug up from an open field in Iwate Agricultural Research Center on November 27, 2014 and had been planted in the heated cultivating beds (length 1.2 m x width 0.8 m x height 0.45 m for each plot) set in a greenhouse at the University field on December, 12, 2014. Thirty to forty rootstocks were planted for one plot (as described in the experimental treatments 2014). Three trial plots with 2 repetitions were prepared as follows: Control; with no supplemental light, FL1-24; continuous (24 hours a day) lighting with 1 white fluorescence lamp (FHF 32EX-N- H, Panasonic Co., Japan), and FL4-24; continuous (24 hours a day) lighting with 4 white fluorescence lamps. All the test plots were covered with a light reflecting material Tyvek sheet (DuPont-Asahi Flash Spun Products Co., Japan) during the night (Fig. 5). The minimum air temperature was kept more than 10°C using an oil heater and soil temperature was kept around 18°C using the heating wires. The trial was conducted from January 16 to February 29, 2015 (harvest period-6 weeks).

Experimental treatments by using winter 'fusekomi' forcing culture on 2016

Preparation for the experiments on 2016 was conducted as follows; one-year-old rootstocks of 'UC157' were dug up from an open field in Iwate Agricultural Research Center on December 1, 2014 and had been planted in the heated cultivating beds (length 1.2 m x width 0.8 m x height 0.45 m for each plot) set in a greenhouse at the University field on December 14, 2015. Thirty to forty rootstocks were planted for one plot (as described in the experimental treatments 2014). Three trial plots with 2 repetitions were prepared as follows: Control; with no supplemental light, FL1-24; continuous (24 hours a day) lighting with 1 white fluorescence lamp (FHF 32EX-N-H, Panasonic Co., Japan), and Blue; continuous (24 hours a day) lighting with 1 blue lamp (FL40S B, Toshiba Co., Japan), UV-B 24; continuous (24 hours a day) lighting with 1 UV-B lamp (G40T10E, Sankyo Denki Co., Japan). All the test plots were covered with a light reflecting material Tyvek sheet (DuPont-Asahi Flash Spun Products Co., Japan) during the night (Fig. 5). The minimum air temperature was kept more than 10°C using an oil heater and soil temperature was kept around 18°C using the heating wires. The trial was conducted from January 17 to February 29, 2016 (harvest period-6 weeks).

Experimental treatments by using winter 'fusekomi' forcing culture on 2017

Preparation for the experiments on 2017 was conducted as follows; one-year-old rootstocks of 'UC157' were dug up from an open field in Iwate Agricultural Research Center on December 7, 2016 and had been planted in the heated cultivating beds (length 1.2 m x width 0.8 m x height 0.45 m for each plot) set in a greenhouse at the University field on December, 9, 2016. Thirty to forty rootstocks were planted for one plot (as described in the experimental treatments 2014). Three trial plots with 2 repetitions were prepared as follows: Control; with no supplemental light, FL4-24; continuous (24 hours a day) lighting with 4 white fluorescence lamps (FHF 32EX-N-H, Panasonic Co., Japan), and UV-B 12; 1 UV-B lamp for 12 h a day (G40T10E, Sankyo Denki Co., Japan). All the test plots were covered with a light reflecting material Tyvek sheet (DuPont-Asahi Flash Spun Products Co., Japan) during the night (Fig. 5). The minimum air temperature was kept more than 10°C using an oil heater and soil temperature was kept around 18°C using the heating wires. The trial was conducted from January 16 to February 27, 2017 (harvest period-6 weeks).

Light intensity and air temperature measurement

Light intensity at night and daytime, and air temperature of the plots were measured during the harvest period. Light intensity was measured under different weather, with

24 cm height from the compost level by using photo quantum meter (Kaito memory sensor MES-136 light logger, Yokohama, Japan). Air temperatures were recorded by using thermo recorder (T&D wireless thermo recorder RTR-52A, Nagano, Japan).

Spear weight observation and spear color analysis

Yield observation was difficult to conducted in this study because of the limited plots, so we used the average of spear weight per plot as the parameter to observed the effect of irradiation time on yield in this experiment, considered spear weight are also an important factor for yields. Spears were harvested at 27 cm and cut to a length of 24 cm for weight observation. The weight of spears in each plot was measured every day during the harvest period. Spear color was analyzed by using the same method as described in the previous chapter.

Determination of rutin contents

For component analysis, rutin content observations were determined. Three to four of M (middle; 13-18 g) to L (large; 18-23 g) sized spears in each plot were cut into 3 sections of equal length (8 cm of each section), immediately frozen, and then lyophilized. Only the top section of the spears was used for rutin analysis (as described in chapter 2). Rutin extraction and determination was conducted using the same method as described in the previous chapter (Maeda et al., 2012; Wambrauw et al., 2016).

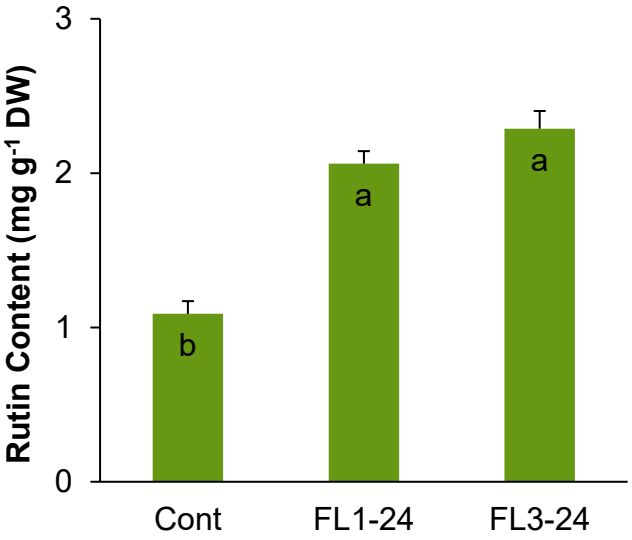

Fig. 12 Effects of irradiation period of supplemental lighting on the rutin contents in the asparagus spears harvested by winter 'fusekomi' forcing cultivation on 2014. Bars indicate SE. Different letters indicate significant differences (Tukey-Kramer test, $P<0.05$, $n=18$)

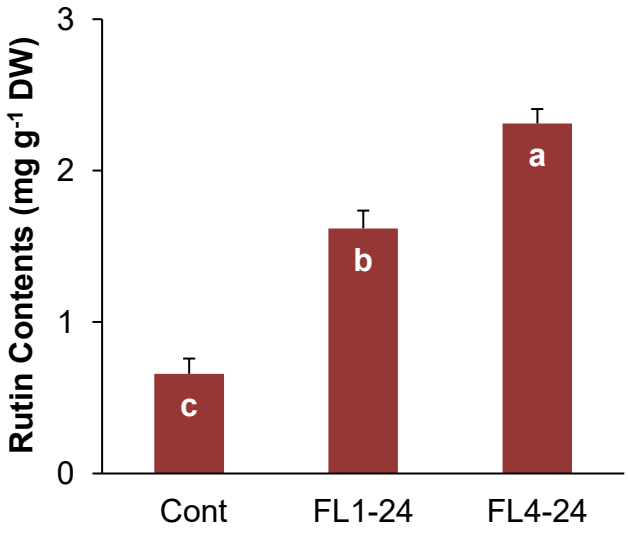

Fig. 13 Effects of supplemental lighting numbers on the rutin contents in the asparagus spears harvested by winter 'fusekomi' forcing cultivation on 2015. Bars indicate SE. Different letters indicate significant differences (Tukey-Kramer test, $P<0.05$, $n=18$)

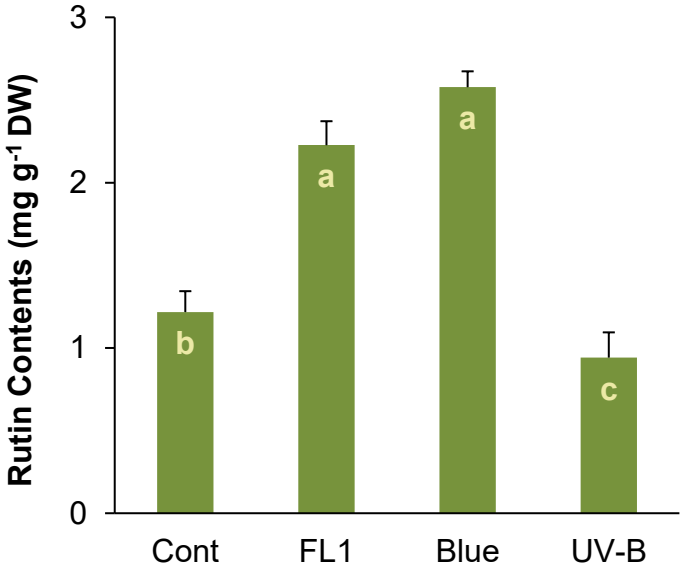

Fig. 14 Effects of supplemental lighting numbers on the rutin contents in the asparagus spears harvested by winter 'fusekomi' forcing cultivation on 2016. Bars indicate SE. Different letters indicate significant differences (Tukey-Kramer test, $P<0.05$, $n=18$)

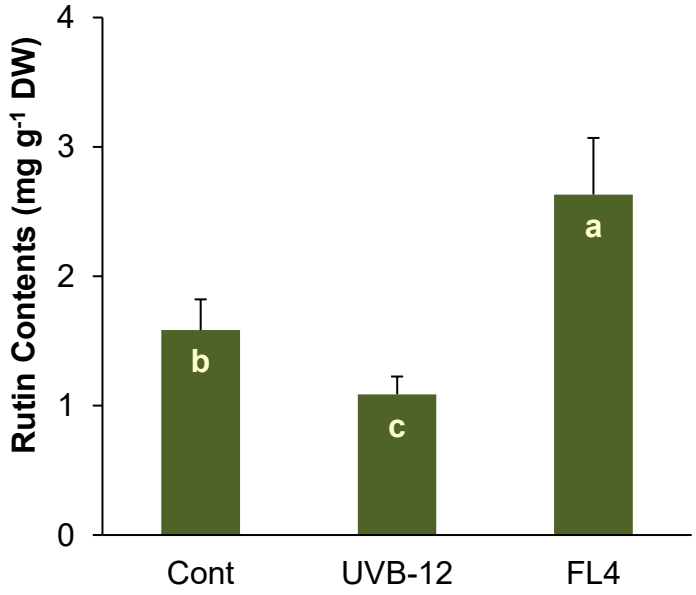

Fig. 15 Effects of supplemental lighting numbers on the rutin contents in the asparagus spears harvested by winter 'fusekomi' forcing cultivation on 2017. Bars indicate SE. Different letters indicate significant differences (Tukey-Kramer test, $P<0.05$, $n=18$)

RESULTS

Effect of different quantity of fluorescent light on rutin content (2014)

During the harvest period rutin content of control was 1.07 mg g^{-1} DW. Rutin content in FL1-24 and FL3-24 plots was 2.06 mg g^{-1} DW, and 2.29 mg g^{-1} DW. Rutin content in both supplemental lighting treatments was observed significantly about twice as many against control (Tukey-Kramer test, $P<0.05$, $n=18$).

Effect of different quantity of fluorescent light on rutin content (2015)

Control plot showed 0.66 mg g^{-1} DW and FL1-24 was observed to contain 1.62 and 2.31 mg g^{-1} DW on the FL4-24, respectively (Fig.13). We confirmed that rutin content was significantly increased in both supplemental lighting plots against control, and even among supplemental lighting plots, there was a significant difference observed (Tukey-Kramer test, $P<0.05$, *n=18)*.

Effect of different quantity and quality of light on rutin content (2016)

The average of rutin content in control plot showed 1.22 mg g^{-1} DW, FL1-24 was observed to contain 2.23 mg g^{-1} DW, blue light was 2.58 mg g^{-1} DW and UV-B 24 was observed to contain mg g^{-1} DW, respectively (Fig.14). We confirmed that rutin content

was significantly higher in both FL1-24 and blue light treatment, while UV-B was the lowest (Tukey-Kramer test, $P<0.05$, $n=18$).

Effect of different quantity and quality of light on rutin content (2017)

We confirmed that UV-B 24 seems to causes stress and damage to spears on the 2016 experiment, to clarify how UV-B effects asparagus spears, in this experiment we changes the irradiation time from 24 h to 12 h. Rutin content was significantly higher in FL4-24, following by control, while UV-B 12 still was the lowest (Fig. 15). FL4-24 was observed to contain 2.63 mg g^{-1} DW; Control plot showed 1.58 mg g^{-1} DW; UV-B 12 was observed 1.09 mg g^{-1} DW, respectively. The same pattern and/or trend was confirmed ; rutin content on UV-B treatments were the lowest, however it was significantly higher in supplemental lighting treatment, FL4-24 and also control as no supplemental lighting treatment (Tukey-Kramer test, $P<0.05$, $n=18$).

Effect of different quantity of fluorescent light on spear color

We used L*a*b* value to observed spear color. L* defines lightness, a* defines red if the measured value are plus (+) and green if the value minus (-), b* defines yellow as the value are plus (+) and blue if it is minus (-). On 2014, compare to the control plots, both supplemental lighting plots (FL1-24 and FL3-24) showed low L* value (Fig.16).

Same trend was also observed in the following year, FL1-24 and FL4-24 showed significantly low against control (Fig.17). Both results also showed that even among the supplemental lighting plots significant differences were observed. However, on 2016, spears grown under blue light was observed much paler compare to the FL1-24 (Fig.20). We found that spears grown under UV-B treatment has low rate of growth, even if its grown, the spear has undesirable spear form, hard, and the color were pale green (Fig.21).

Effect of different quantity of fluorescent light on spear weight (2014 and 2015)

The yield observation was conducted every day during harvest period, we measured the weight of spear and took average only from the spear over 8 g and under 23 g. The observation of spear weights was only conducted on 2014 and 2015. The average of spear weight on 2014 of control plot, FL1-24 and FL3-24 plot were 14.5 g, 14.5 g, and 13.9 g, respectively (Fig. 22). No significant difference was observed between control and FL1-24 plot on both 2014 and 2015, however, there was a significant difference between control against FL3-24 and FL4-24. Compare to the average of spear weight on control, FL3-24 spear became a bit lighter (Tukey-Kramer test, $P<0.05$, $n=420$). Same trend was also found on 2015 test. The average of spear weight on control plot, FL1-24 and FL4-24 plot were 16.5 g, 16.0 g, and 13.9 g, respectively (Fig.23). The spears on

supplemental lighting treatment become lighter compare to those in the control plots (Tukey-Kramer test, $P<0.05$, $n=488$).

DISCUSSION

We found that L* value of the spear harvested at supplemental lighting plots was significantly lower compare to the control, even within the supplemental lighting plots, as the number of lamps getting higher. L* value defines brightness of the color. If the values near hundred, the color is close to white, in contrast, if the values are low and/or near zero, the color is relatively dark. The reconstructed color of each treatment becomes better according to number of lamps. Results of this study suggested that the color of the spears grown under the supplemental lighting became deeper than the control plots in association with numbers of the fluorescent lamps, and it means to be better color (Fig.18, Fig.19). Interestingly, spear color in blue light treatment was much paler compare to the FL1-24 h plot; indicated that, blue light had no effects on enhancing chlorophyll content (Fig.20).

The investigation of rutin content by using different quantity and quality of fluorescent light shows that rutin was increased significantly by the presence of fluorescence lamps. On 2014, rutin content on FL1-24 and FL3-24 h treatment

significantly increased against control. The same trends also were observed on 2015 trial. Rutin content on FL1-24 and FL4-24 h treatment significantly increased against control plot. Moreover, rutin content of FL4-24 were significantly high against FL1-24; suggesting that as the number of lamps increases, rutin contents will also gradually increase.

In the study of the effects of light quality on rutin content, we found that, spears in the blue light for 24 h plot, following by FL1-24 h plot has the highest rutin content against control. Both blue light for 24 h plot and FL1-24 h plot showed similar tendency (Blue: 2.58 mg g^{-1} DW; FL1: 2.23 mg g^{-1} DW), statistically no significant difference was observed. However, spears grown on the blue light had paler color compare to the FL1 plot; suggesting that blue light only works to enhance rutin biosynthesis (flavonoid biosynthesis) but not on chlorophyll. The same results were also reported that buckwheat grown under blue light has higher rutin content than under natural light (Lee et al., 2001). Another studies also indicate that flavonoid related gene expressions was increased significantly when it exposed to blue light combine with UV-B and white light (Ebizawa et al., 2008; Ahn et al., 2015). These studies support our results that blue light work specifically to stimulate rutin synthesis and might have relation with photoreceptors system (Campbell et al., 1976; Suzuki et al., 1987; Hofmann et al., 2000,

2001, 2003; Batschauer et al., 1996). UV-B causes severe damage to spears, such as the color was much paler, spears got harden and malformed. It seems like the dozes of UV-B we used in this study were too much and it leads to cause severe damages to the spears, and resulted in the low rutin content on both 24 h and 12 h UV-B treatments. It has been reported that UV-B alone without synergistically work with another light such as blue light and/or white lamps shows no effects on flavonoid biosynthesis regulation (Ebizawa et al., 2008). The addition of UV-B with small amount of dozes and in some prior irradiation time is believed to work to enhance rutin (Goto et al., 2016). So, it is thought that the light intensity, the dozes and irradiation time play an important role in this case. Many studies had been reported about the relations between light and rutin (flavonoids). It was suggested that both have special relation because light is the most important factor to enhance flavonoid regulation. Studies about the relationship between light and flavonoid have been studied well and still ongoing (Kubasek et al., 1992; 1998; Wade et al., 2001; Rozema et al., 2002). Previous studies in asparagus, buckwheat, and arabidopsis showed that rutin contents got higher under light treatment (Maeda et al., 2010; Wambrauw et al., 2016; Lee et al., 2001; Kreft et al., 2002; Li et al., 2010; 2012).

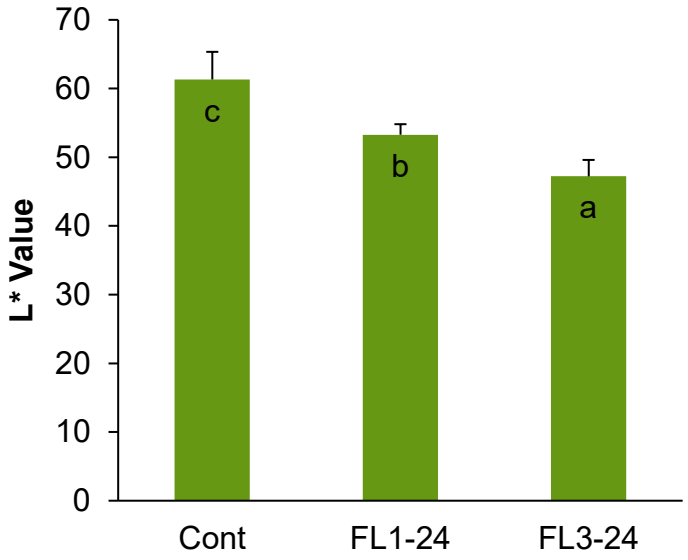

Fig. 16 Effects of supplemental lighting on color of the spears (L* value, measured by a color difference meter). (2014). Bars indicated SE. Different letters indicated significant differences (Tukey-Kramer test, $P<0.05$, $n=24$).

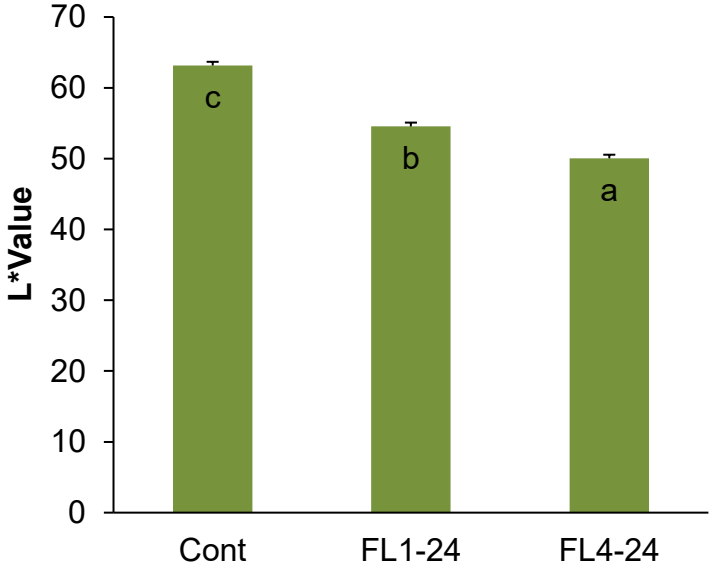

Fig. 17 Effects of supplemental lighting on color of the spears (L* value, measured by a color difference meter). (2015). Bars indicated SE. Different letters indicated significant dif-ferences (Tukey-Kramer test, $P<0.05$, $n=24$).

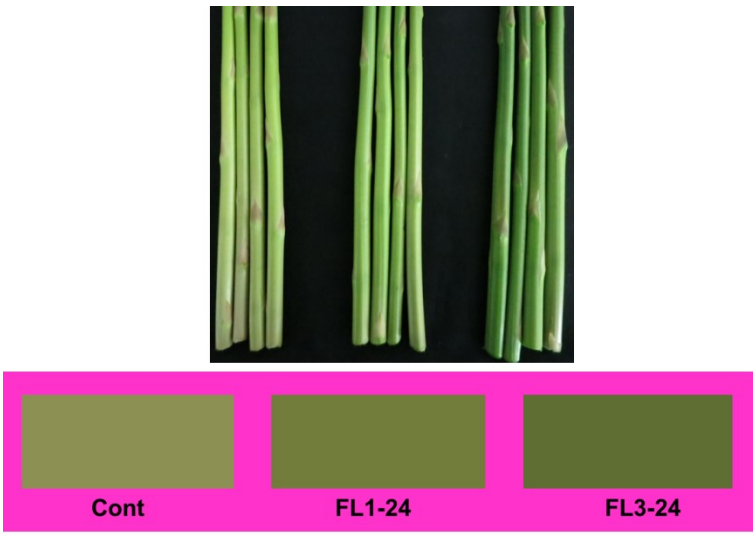

Fig. 18 Differences in spear color harvested on 2014 trial and the reconstructed color of L*a*b* average values (*n*=24) by using the software 'Irodashimeijin for windows'.

Fig. 19 Differences in spear color harvested on 2015 trial and the reconstructed color of L*a*b* average values (*n*=24) by using the software 'Irodashimeijin for windows'.

Fig. 20 Spears grown under blue light 24 h treatment (left) and FL1-24 treatment (right) on winter fusekomi forcing cultivation (2016).

Fig. 21 Spears grown under UV-B treatment

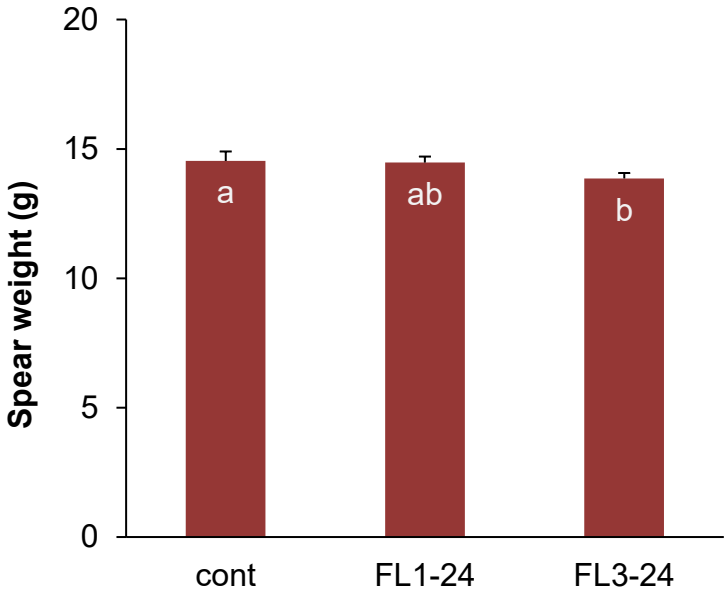

Fig. 22 Effects of supplemental lighting on the average spear weight of the asparagus spears harvested by winter 'fusekomi' forcing cultivation on 2014. Bars indicated SE. Different letters mean significant differences (Tukey-Kramer test, $P<0.05$, $n=420$).

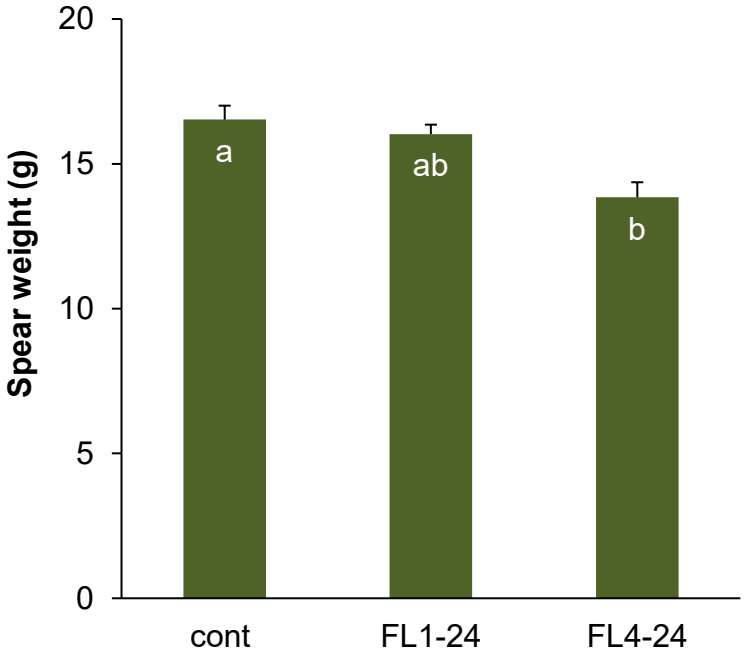

Fig. 23 Effects of supplemental lighting on the average spear weight of the asparagus spears harvested by winter 'fusekomi' forcing cultivation on 2015. Bars indicated SE. Different letters mean significant differences (Tukey-Kramer test, $P<0.05$, $n=488$).

The relative expression of flavonoid biosynthetic genes in lecctuce and arabidopsis also showed that flavonoid related genes were effectively express by the induction of blue light and UV-B light (Kubasek et al., 1992; Fuglevand et al., 1996, Ebizawa et al., 2008). These studies demonstrated that rutin content tends to fluctuate according to the light environment. Light intensisty, light quantity, and light quality have deep relation with the increasing of rutin synthesis; rutin content getting higher due to the quantity of supplemental lighting and blue light work specifically to increase rutin. Therefore this result suggested that supplemental lighting and blue light are found effective to enhanced rutin biosynthesis in asparagus.

There was severe negative impact on spear weight of spears grown under FL3 and FL4-24 h plots; the spears were significantly lighter than spears in the control plot. It is thought that during the harvest period, except the control plots, spears grown in the FL treatment were irradiated almost 6 week under 24 h and it affected directly to the air temperatures inside the tunnels. The air temperatures were about 6°C higher in the continuous supplemental lighting compare to the control. So, these might be the factor of dryness of the compost during examination, therefore affect the spears growth and also spear weight. Further studies are needed in order to find the best condition and also to see the limitation of the light effects to asparagus spears. We have worked on how

light and rutin relate in the rutin content. Unlike others plant, the enzymes involved in flavonoids biosynthesis pathways on asparagus have not been identified yet. Particular studies about mechanism of the rutin increased by supplemental lighting in asparagus from molecular biology aspect are needed.

As the conclusion, the results of this study showed that supplemental lighting with different quantity of lamps and blue light was effective to improve the quality of asparagus spears, especially for the area that has low sunlight in winter season. And beside it is easy to conduct and could help to improve spear quality, it also could keep temperature inside the tunnel warm. Blue light combine with white fluorescent lamps could be one of the candidates to control rutin regulation artificially in order to produce high quality of asparagus spears.

CHAPTER 4

4.1. Gene Expression of Flavonoid Biosynthetic Genes in Green and White Asparagus Spears

INTRODUCTION

Studies continues to be made in order to understand the roles of flavonoids in stress protection, as well as in defining the mechanisms that control the amounts and varieties of flavonoids that are produced in plants in response to diverse environmental cues (Shirley et al., 2002 ; Chalker-Scott., 1999). The amount of rutin was getting higher and the spear color are also getting better by introducing supplemental lighting (Wambrauw et al., 2016), and in the previous studies we also showed that rutin is getting higher according to the number of lamps and light quality (blue light). However there is still a few studies on molecular regulation of flavonoid metabolism in asparagus; how light effects the accumulation of rutin and which genes in the flavonoid biosynthesis pathway are light related gene and/or the light response genes to enhance rutin production. Thus the aim of this study is to investigate the effect of light on rutin biosynthetic genes in asparagus spears by examined green spears; spear which grown in the natural sunlight and white spears; sunblocked asparagus.

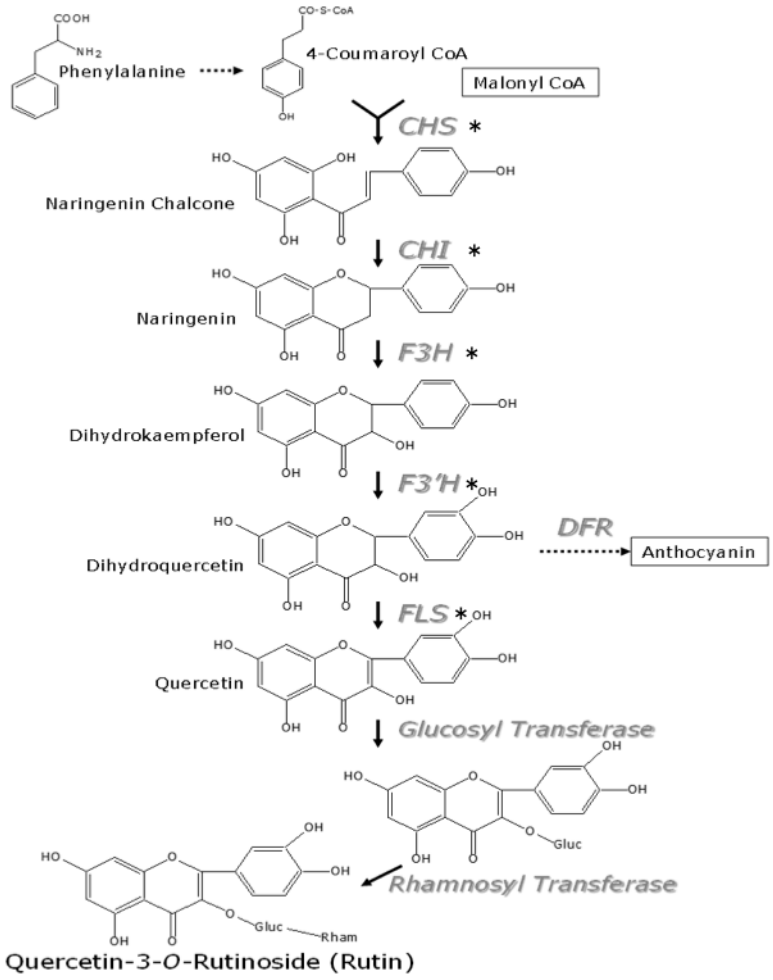

Fig. 24 Schematic of flavonoid biosynthesis in Asparagus. Two general classes of end product are produced in Asparagus: Flavonols (Rutin:Quercetin-O-Rutinoside), and Anthocyanins. Enzymes are indicated in bold and italic: CHS, chalcone synthase (CHS catalyses the first committed step in this pathway) ; chalcone isomerase, CHI; flavone synthase; F3H, flavanone 3-hydroxylase; F3'H, flavonoid3'- hydroxylase; DFR, dihydroflavonol 4-reductase; FLS, flavonol synthase ; glucosyl transferase, GT ; rhamnosyl transferase, RT. Genes with * is the genes examined in this study.

MATERIALS AND METHODS

Plant Material

Green and white asparagus spear were used as the plant material. The sample for the first examination was took at Hirosaki University during the harvest period of winter 'fusekomi' forcing culture. One-year-old rootstocks of 'UC157' were dug up from an open field in Iwate Agricultural Research Center on November 19, 2013. The rootstocks had been planted in the heated cultivating beds (length 1.2 m x width 0.8 m x height 0.45 m for each plot) set in a greenhouse at the University field on December 6, 2013. Thirty to forty rootstocks were planted for one plot (80 cm x 120 cm x 45 cm), and were covered with rice hull compost. The entire test plots for green spear were covered with the plastic tunnels and the house was covered with shading net. White spears were covered with a sun blocking film, White Silver, TOKANKOSAN CO, LTD, Japan. The minimum air temperature was kept higher than 10°C using an oil heater and soil temperature was kept around at 18°C using the heating wires. The trial was conducted from January 14 to February 24, 2014 (harvest period-6 weeks). The second sample was collected from Farm Horo, Shin Hidaka-Cho, Hokaido, Japan. Both green and white spears were harvested in June, 2016 during spring harvest season from 4 years old plants of UC-157 grown in the green houses. To produce white asparagus, spears were

covered with White Silver film. (TOKANKOSAN CO, LTD).

Determination of Rutin Contents

Three to four of M (middle; 13-18g) to L (large; 18-23g) sized spears were cut into 3 sections of equal length (8 cm of each section), immediately frozen, and then lyophilized. Only top sections of the spears were used for rutin analysis (As described in chapter 2). The extraction and determination of rutin were conducted using same method as described in chapter 2.

Gene Expression Analysis

Sample for total RNA extraction was took from the same sample described above (Farm Horo and Hirosaki University). The total RNA was extracted by using RNeasy Plant Mini Kit (QIAGEN). About 50-100 mg of epidermal tissue was collected from the tip of the spear then used for extraction. The cDNA was reverse-transcribed from 1 μg of extracted total RNA, using QuantiTech Reverse Transcription Kit (QIAGEN). The gene expression levels were evaluated by using real-time polymerase chain reaction (real-time PCR) methods with DNA ENGINE OPTICON 2 (BIO-RAD, USA). All target gene primer and reference gene primer (*Actin*) were shown on Table 2. The real-time PCR was performed by using FastStart Universal SYBR Green Master (Rox) (ROCHE, USA). The optimized reaction was carried out in 20 μl reaction system

containing, 10 µl of FastStart Universal SYBR Green Master (Rox) supplied master mix, 0. 12 µl of each primers (50 pmol), 2 µl (20ng) template cDNA, and 7.76µl of sterile water. PCR protocol was as follows: denaturation for 10 min at 95°C, followed by 40 cycles of denaturation for 10 s at 95°C, annealing for 30 s at 60°C, Plate Read at 78°C for 2 min, Plate Read go to line 2 for 39 cycles. Melting curve 60 °C - 78°C. PCR results were calculated as the mean of 3 replicated treatments.

RESULTS

Rutin was not detected on white spears whereas light exposed green spears has higher rutin content, 3.2 mg g^{-1} DW (Fig. 25). To investigate how light influence the regulation of flavonoid related genes, in the first experiment, total RNA was extracted from the same spears (green and white spears) used for rutin analysis (Fig. 26; Fig. 27). The first gene on the flavonoid biosynthetic pathway, *Chalocone synthase* (*CHS*) has no difference in its relative gene expression. Same trend was also found in *Chalcone Isomerase* (*CHI*) there was no significant difference among green and white (Fig.26). *Flavanone 3-hydroxylase (F3H)* shows no significant difference between green and white, although, its relative gene expression is seems decreased in white spears.

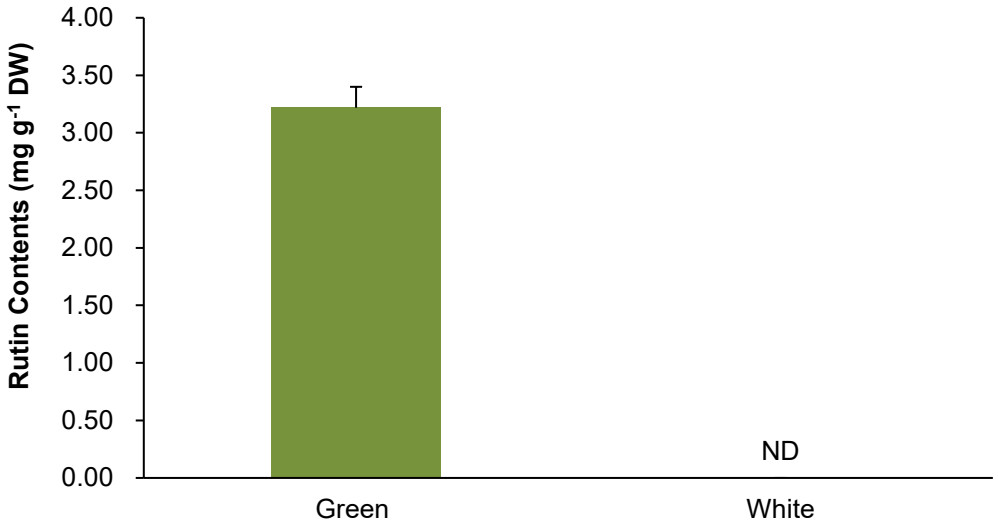

Fig. 25 Rutin content of green and white spears. Green spears are spear grown under natural light and white spears are spears grown under dark condition. Bars indicated SD (*n*=12). ND represented not detected

Flavanone 3'-hydroxylase (F3'H) expression were significantly higher in green than lower in white. The relative gene expressions of *Flavonol Synthase (FLS)* were also significantly higher in green asparagus compare to white (Fig.27). The expression pattern of the second sample was found similar as the first sample; the earliest genes and/or upstream genes (*CHS* and *CHI*) has similar expression level in both sample (data not shown). However, the expression of the downstream genes, especially *FLS* was found significantly higher in green than in white (Fig.28).

DISCUSSION

The comparison of rutin content and rutin related gene expression on green and white spear was conducted. Light is one of the most important factors to enhance flavonoid biosynthesis (Wade et al., 2001; Jenkins et al., 2001; Fuglevand et al., 1996). Flavonoid was reported to fluctuate with different light quality and quantity. (Stracke et al., 2007; Pelletier et al., 1999, Ebizawa., 2008; Lee et al., 2001). By the presence of light, rutin content was getting higher, importanly, rutin could be articificially controled by changing the light environment (Wambrauw et al., 2016). However, there is limited information on the molecular regulation of the flavonoid metabolism in asparagus. In order to clarify the relationship between rutin biosynthesis and light exposure, we used green (light exposed) spears and unpigmented white spears (light-shielded green spear) to see rutin related gene expression.

As shown in the first experiment (Fig. 25), white spear has no rutin same as former report (Maeda et al., 2005). In contrast, light exposed green spear has higher rutin content, 3.2 mg g^{-1} DW. *Chalocone synthase* (*CHS*), the first gene on the flavonoid biosynthetic pathway, following by *Chalcone Isomerase* (*CHI)* and *Flavanone*

3-hydroxylase (*F3H)* were found no significant differences between green and white spears. The upstream genes of the flavonoid pathway in asparagus were expressed in unpigmented white spear almost similar with green spear. It is though that, the expression of upstream flavonoid gene in unpigmented white spear might involve in synthesis of other secondary metabolites. This fact may explain why these genes expressed in the absences of rutin synthesis in white spears (Boss et al., 1996). However as we go down to the downstream genes on the flavonoid pathway, *flavanone 3'-hydroxylase (F3'H)* and *flavonol synthase (FLS)*, the relative gene expressions were found significantly lower in the white and high in green.

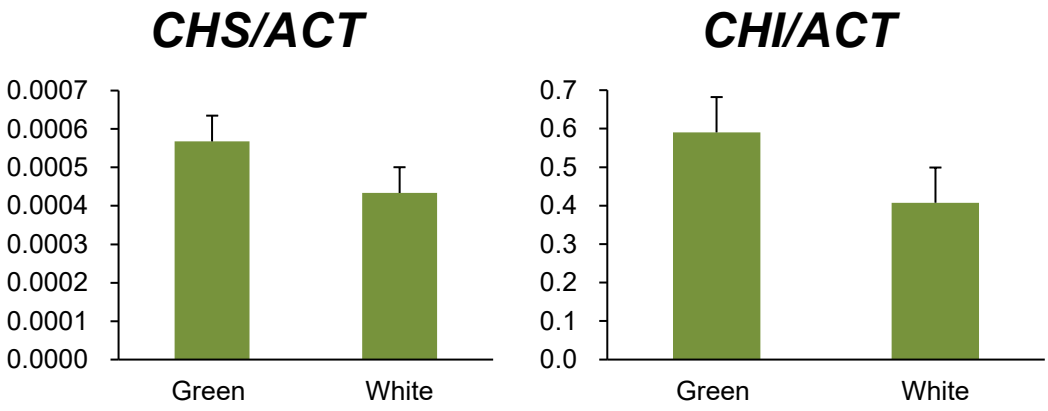

Fig.26 Expression level of the earliest genes in flavonoid biosynthesis pathways; *CHS , chalcone synthase* and *CHI, chalcone isomerase.* Green spears are the spears grown under natural light and white spears are the spears grown under dark condition. Both Green and white spears were harvested during harvest period of winter fusekomi forcing culture at Hirosaki University. Bars indicated SD (*n*=3). Statistical data was performed by t-test * $p<0.05$.

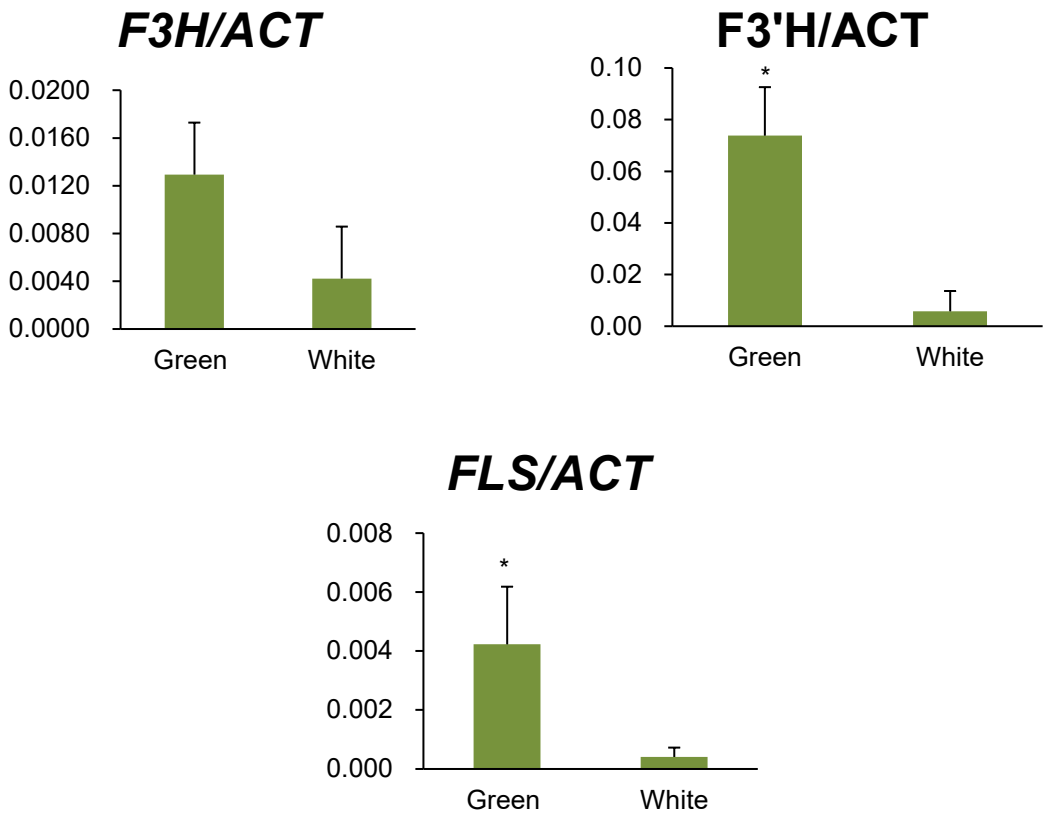

Fig.27 Expression level of the downstream genes in flavonoid biosynthesis pathways; F3H, *flavanone 3-hydroxylase*; F3'H, *flavonoid 3'-monooxygenase*; FLS, *flavonol synthase*. Green spears are the spears grown under natural light and white spears are the spears grown under dark condition. Both green and white spears were harvested during harvest period of winter fusekomi forcing culture at Hirosaki University. Bars indicated SD (*n*=3). Statistical data was performed by t-test * $p<0.05$.

The expression pattern was consider vary according to the plant; for example, expression of all flavonoid genes in maize is induced when tissues pigmentation began, whereas in petunia and snapdragon, their start point are the downstream genes in the pathway beeing, *DFR* and *F3H*. In buckwheat and lettuce, *FLS*, the downstream genes were upregulated in the present of light than in the dark (Martin et al., 1993; Li et al., 2012; Ebizawa et al., 2007).

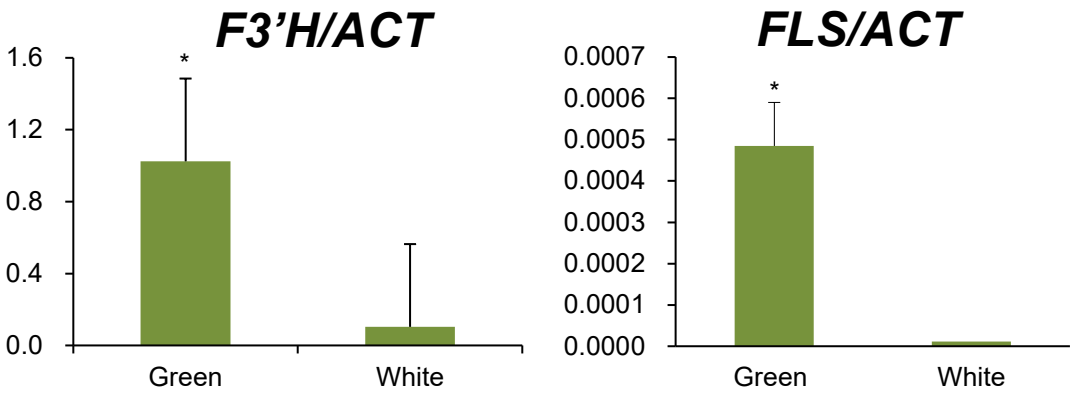

Fig.28 Expression level of the latest genes in flavonoid biosynthesis pathways; F3'H, *flavonoid 3'-monooxygenase*; FLS, *flavonol synthase*. Green spears are the spears grown under natural light and white spears are the spears grown under dark condition. Both green and white spears were harvested during spring harvest period in Farm Horo, Hokaido, Japan. Bars indicated SD (n=3). Statistical data was performed by t-test * $p<0.05$.

In this study, we found that as it goes to the downstream, the expression of flavonoids genes, especially *FLS* in white spear as unpigmented spears are gradually decreased; the expression level were found almost drop to zero. *FLS* expression pattern in both, first sample and second sample were lower than in *F3'H* and *FLS* expression itself was quite lower even in the green spears (Fig.27, Fig.28). This result indicates that *FLS* responded to light; if there is no light, their expression were almost drop to zeros, however by the presence of light *FLS* expression level were increased in green as described in this studies. A number of former studies indicated that *FLS* catalyzes the reaction from dihydroquercetin to flavonol (quercetin/ rutin) are the key enzyme in the rutin biosynthesis (Holton et al., 1993; Pelletier et al., 1999; Wisman et al., 1998;

Stracke et al., 2007). Studies in buckwheat and lettuce show that *FLS* was affected by light (Ebizawa et al., 2007; Li et al., 2010). These report supported our finding that *FLS* needed light and *FLS* is an important enzyme which relates to react to light exposure, and enhance rutin biosynthesis, in other words, indicating *FLS* is the key enzyme in rutin synthesis.

We investigated the relation of light exposure and the gene expression of flavonoid related genes in asparagus. Our studies indicating that light enhance rutin biosynthesis and *FLS* play an important role in response to light in asparagus.

4.2. The Effect of Light Intensity on Asparagus Flavonoid Biosynthetic Gene Expression

INTRODUCTION

The results of our previous study in chapter 3, 2015 examination indicated that rutin content was significantly higher in FL4-24 h treatment than in control (no supplemental lighting treatment). According to the number of lamps, rutin content was getting higher. In the previous section, studies about gene expression of flavonoid related genes in light exposed green spear and the unexposed light white spear was conducted. According to these studies, the downstream gene, *FLS* was light related gene in flavonoid biosynthesis pathway. So in this section, in order to clarify the mechanism of how light intensity effect flavonoid related genes expression; the same sample used in 2015 examination also have been used in this section.

MATERIALS AND METHODS

Plant Material

Spear used in this test was as follows; White: unpigmented spears; Green: Green asparagus with no supplemental light; FL1: Spears grew under continuous (24 hours a day) lighting with 1 white fluorescence lamp; FL4-24; continuous (24 hours a day)

lighting with 4 white fluorescence lamps. The sample preparation for the experiments on 2015 was conducted as described in chapter 3, "Experimental treatments by using winter ‘fusekomi’ forcing culture on 2015”.

Gene Expression Analysis

The total RNA was extracted by using RNeasy Plant Mini Kit (QIAGEN). About 50-100 mg of epidermal tissue was collected from the tip of the spear then used for extraction. The cDNA were reverse-transcribed from 1 μg of extracted total RNA, using QuantiTech Reverse Transcription Kit (QIAGEN). The gene expression level was evaluated by using real-time polymerase chain reaction (real-time PCR) methods with DNA ENGINE OPTICON 2 (BIO-RAD, USA). The real-time PCR was performed by using FastStart Universal SYBR Green Master (Rox) (ROCHE, USA). All target gene primer and reference gene primer (Actin) was shown in table 2. The PCR reaction and protocol was as described in section 4.1.

RESULTS

All genes of the enzymes involved in the asparagus flavonoid biosynthesis pathway; *chalcone synthase* (*CHS*), *chalcone isomerase* (*CHI*), *flavanone 3-hydroxylase* (*F3H*), *flavonoid 3'- hydroxylase* (*F3'H*), *flavonol synthase* (*FLS*) were expressed in white,

green control (no light treatment), FL1 and FL4, respectively. The expression of the upstream genes, *CHS* expression level was high in white while the expression in green, FL1 and FL4 was lower. In contrast, *CHI* and *F3H* expression level was higher in FL4 than other treatment. While *F3'H* expression showed no difference in white and FL4, their expression was low and high in Green and FL1. The expression level of *FLS* in white spears was significantly lower compared to FL4. On the contrary, FL4 expressions were significantly high against white and other treatment. There is no significant difference between green control and FL1 (Fig.29; Fig. 30).

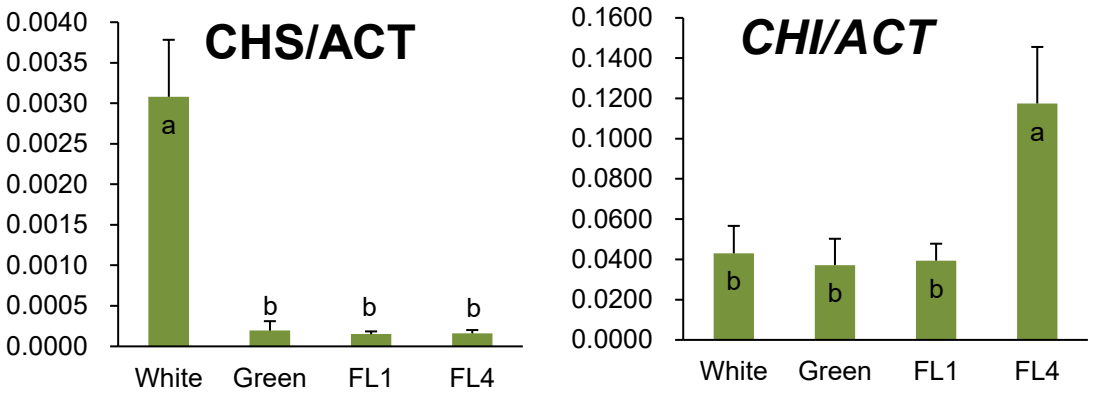

Fig. 29 Expression level of the upstream genes in flavonoid biosynthesis pathways; *CHS, chalcone synthase* and *CHI, chalcone isomerase.* White: unpigmented spears; Green: Green asparagus with no supplemental light; FL1: Spears grown under continuous (24 hours a day) lighting with 1 white fluorescence lamp; FL4-24; continuous (24 hours a day) lighting with 4 white fluorescence lamps. Bars indicated SD (*n*=3).

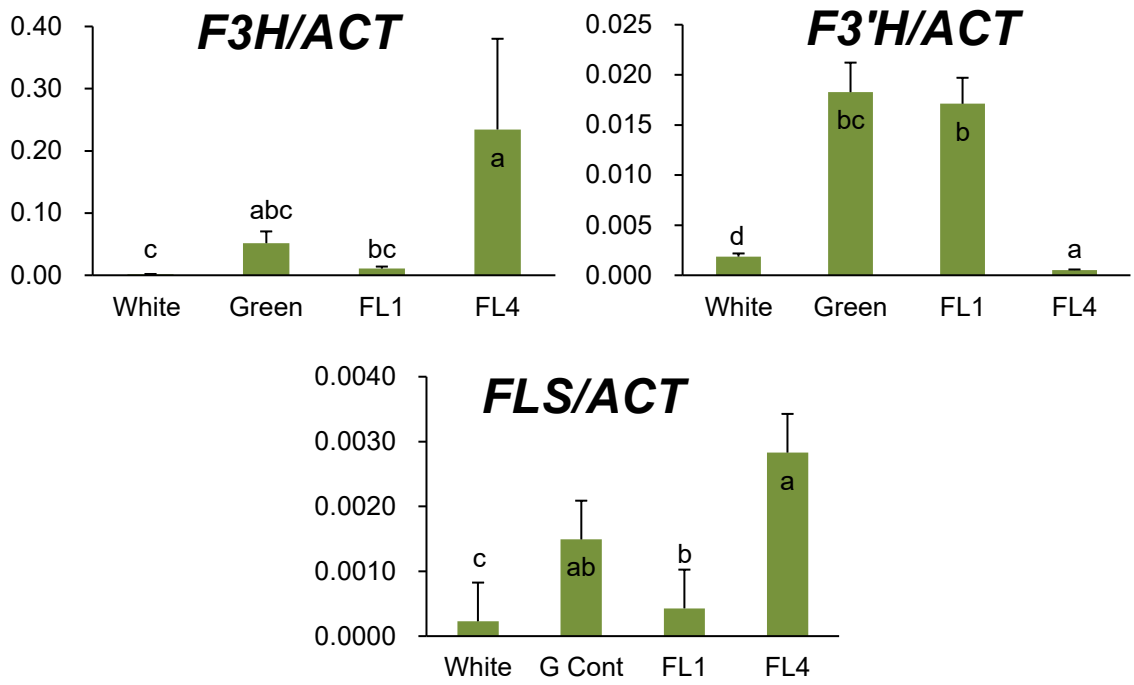

Fig. 30 Expression level of the downstream genes in flavonoid biosynthesis pathways; F3H, *flavanone 3-hydroxylase*; F3'H, *flavonoid 3'-monooxygenase*; FLS, *flavonol synthase.* White：unpigmented spears; Green: green asparagus with no supplemental light; FL1: Spears grown under continuous (24 hours a day) lighting with 1 white fluorescence lamp; FL4-24; continuous (24 hours a day) lighting with 4 white fluorescence lamps. Bars indicated SD (*n*=3).

DISCUSSION

According to the studies in chapter 3, rutin content was significantly increased in both supplemental lighting (FL1-24; FL4-24) plots against control. Control plot showed 0.66 mg g^{-1} DW and FL1-24 plot was observed to contain 1.62 and 2.31 mg g^{-1} DW on the FL4-24 plot, respectively (Fig.13). In this experiment, *CHS* expression level was high in white, while *CHI* expression was high in FL4. The expression level of *F3H* was

higher in FL4 while *F3'H* was high in both green and FL1. Although only CHS that has high expression in white, the rest of the genes have relatively lower expression in white compare to other treatments. We observed that there is no particular pattern in the expression of these genes in all treatment used in this experiment especially in light treated spears; their expression level was erratically varied. Additionally, in our preliminary study, except *FLS* the expression level of the flavonoid related genes is either low or high but no particular pattern was observed. In the previous section, rutin was only found in light exposed green spear but none in the unpigmented white spear. And the gene expression results indicated that *FLS* is light related gene because of its expression level were the lowest in the white and by the presence of light (light exposed green spear), the expression was enhanced. Similar results also were found in this experiment, the expression level of *FLS* was significantly lower in white compared to FL4. And there is no significant difference between green and FL1. FL4 has the highest expression level in *FLS* against other tested plots. According to the light intensity (number of lamps), *FLS* were regulated and/or enhanced. This result indicated and supports our statement that, *FLS* is lightly related gene and plays an important role in flavonoid regulation in asparagus.

4.3. The Effect of Light Exposure Time on Asparagus Flavonoid Biosynthetic Gene Expression

INTRODUCTION

Studies continues to be made in order to understand the roles of flavonoids in stress protection, as well as in defining the mechanisms that control the amounts and varieties of flavonoids that are produced in plants in response to diverse environmental cues (Shirley et al., 2001, 2002). Flavonoids play important roles to protect plant tissue against harmful rays and light is one of the most important environmental signals regulating flavonoid biosynthesis (Rozema, et al.,2002; Gao et al, 1994; Wade et al., 2001; Jeckins, 1997; Fuglevand et al., 1996; Wambrauw et al., 2016). However there are still a few studies on the molecular regulation of flavonoid metabolism in asparagus. In the first and second section of this chapter, we have discussed the results of flavonoid gene expression on green and white asparagus, and also the effect of different light intensity and/or number of lamps to the flavonoid gene expression. *Flavonol Synthase* (*FLS),* the downstream gene in flavonoid biosynthesis pathways showed as light related gene and/or the light response genes to enhance flavonoid regulation. The expression level was relatively lower in white spears compare to other genes because white spears do not contain rutin. In this section, to understand the mechanism of rutin

regulation and its relationship with light, we made white asparagus first by using sunblocking film, and open the sunblock film in order to expose the spear to the natural sunlight until the white spear turned to be green. Samples were harvested in particular time, and then we conducted time-course gene expression analysis about *FLS* gene at first.

MATERIALS AND METHODS

Plant Material

Preparation for the experiments on 2017 was conducted as follows; one-year-old rootstocks of 'UC157' were dug up from an open field in Iwate Agricultural Research Center on December 7, 2016 and had been planted in the heated cultivating beds (length 1.2 m x width 0.8 m x height 0.45 m for each plot) set in a greenhouse at the University field on December, 9, 2016. Thirty to forty rootstocks were planted for one plot (80 cm x 120 cm x 45 cm), and were covered with rice hull compost. All the test plots were covered with the White Silver film to make white spears (TOKANKOSAN CO, LTD). The minimum air temperature was kept higher than 10°C using an oil heater and soil temperature was kept around at 18°C using the heating wires.

Plot Setting

The trial was conducted from January 16 to February 27, 2017 (harvest period-6 weeks). During the harvest period, after spears become white, the sunblock film was uncovered, and the spears were exposed to the natural sunlight. In particular of time the spears were harvested; 0 hours after open (white spears): 0 h, 4 h after open: 4 h, 8 hours after open : 8 h, 16 hours after open : 16 h, 24 hours after open : 24 h, 32 hours after open : 32 h, 40 hours after open : 40 h, 48 hours after open: 48 h, 72 hours after open: 72 h, 96 hours after open: 96 h, 144 hours after open: 144 h, and 1 week after open: 1 W. The same samples were used for both rutin analysis and gene expression analysis.

Determination of Rutin Contents

Three to four of M (middle; 13-18g) to L (large; 18-23g) sized spears were cut into 3 sections of equal length (8 cm of each section), immediately frozen, and then lyophilized. Only top sections of the spears were used for rutin analysis (As described in chapter 2). The extraction and determination of rutin was conducted using the same method as described in chapter 2.

Gene Expression Analysis

Spears used in this experiment were also used for rutin analysis. Spears were harvested at; 0 hours after open (white spears): 0 h, 4 h after open: 4 h, 8 hours after open : 8 h, 16 hours after open : 16 h, 24 hours after open : 24 h, 32 hours after open : 32 h, 40 hours

after open : 40 h, 48 hours after open: 48 h, 72 hours after open: 72 h, 96 hours after open: 96 h, 144 hours after open: 144 h, and 1 week after open: 1 W. Spears were immediately frozen after harvested each time and the total RNA was extracted by using RNeasy Plant Mini Kit (QIAGEN). About 50-100 mg upper parts (8 cm from the tip) of spear tissue were used for the extraction. The cDNA were reverse-transcribed from 1 μg of total RNA using QuantiTech Reverse Transcription Kit (QIAGEN). The gene expressions level was evaluated by using real-time polymerase chain reaction (real-time PCR) methods with DNA ENGINE OPTICON 2 (BIO-RAD, USA). The real-time PCR were performed by using FastStart Universal SYBR Green Master (Rox) (ROCHE,USA). All target gene primer and reference gene primer (*Actin*) were shown on table 2. The PCR reaction and protocol was as described in the section 4.1.

RESULTS

To investigate the relationship between flavonoid biosynthesis and light, the sample where harvested at 0 hour after open (white spears) until 1 week after open (spears become greener) (Fig.31, Fig.34). The samples collected were used for rutin analysis and flavonoid gene expression analysis (*Flavonol Synthase*).

Table 2 Primer Sequences for real-time PCR Analysis

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
AoActin	ATGGGGGCAGAAGGATGCCCTATG	CCACATCTGTTGGAATGTGCT
AoCHS	TGTACCAGCAAGGCTGCTTCG	CTGAATGTGATCCTCACACCG
AoCHI	AAGGGGAAGGATGCTGAGGAG	TAGGTTCCGATGGCTTTCCAG
AoF3H	CTTCAGGGTGAAGCAGTGCAA	CATGGCCTCGGAGAGAACCAC
AoF3dH	GGTGACTTCGTGCCTGCACTGAG	CCTCCTTCAACCTCATCAACACAC
AoFLS	CTCACCACCAGGTGAGCTCG	CTGGGGAGCGATCAAACAATG

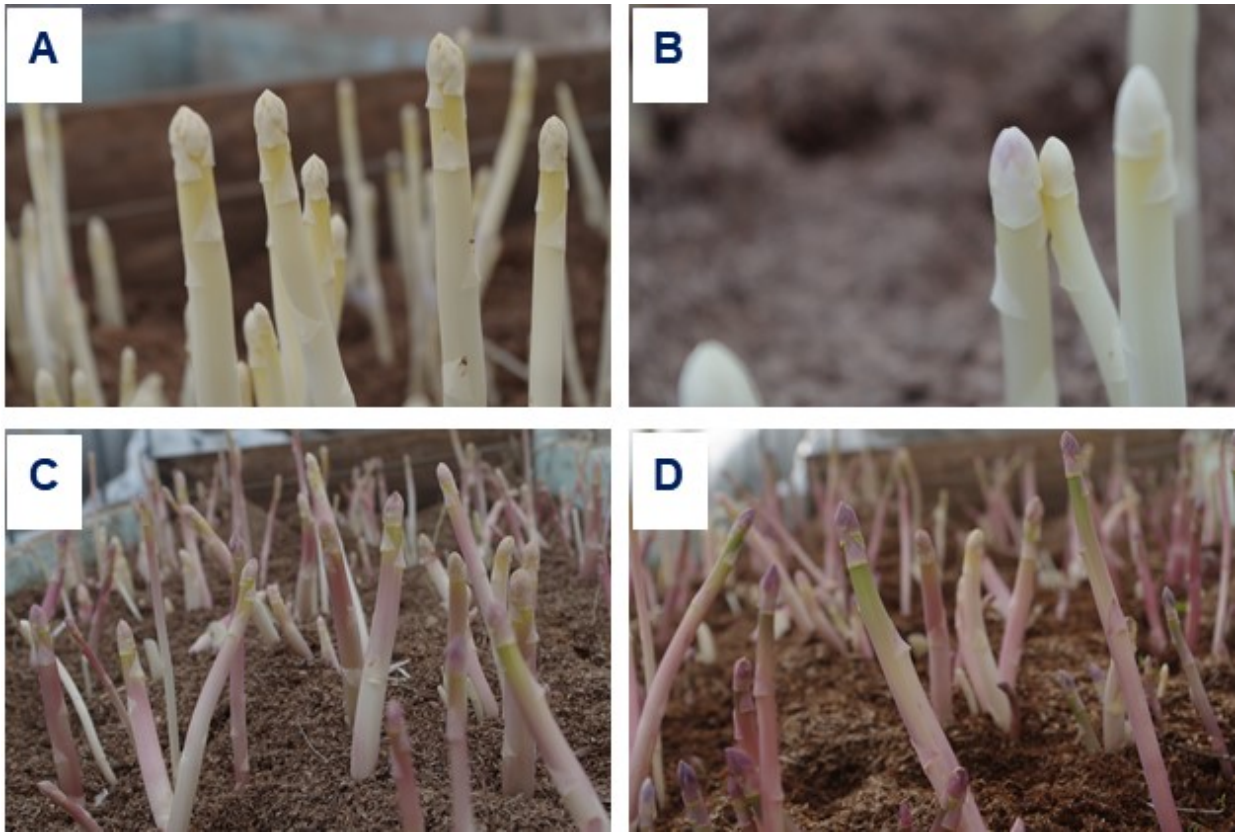

Fig. 31 Spears grown under the natural light during winter fusekomi forcing culture after sunblock film was uncovered ; A : White spear after open (AO) 0 h; B: Spears starts to coloring at 8 h after light exposure: C: Spears condition before harvested at 96 h after light exposure; D: Spears condition before harvested at 1 W after light exposure.

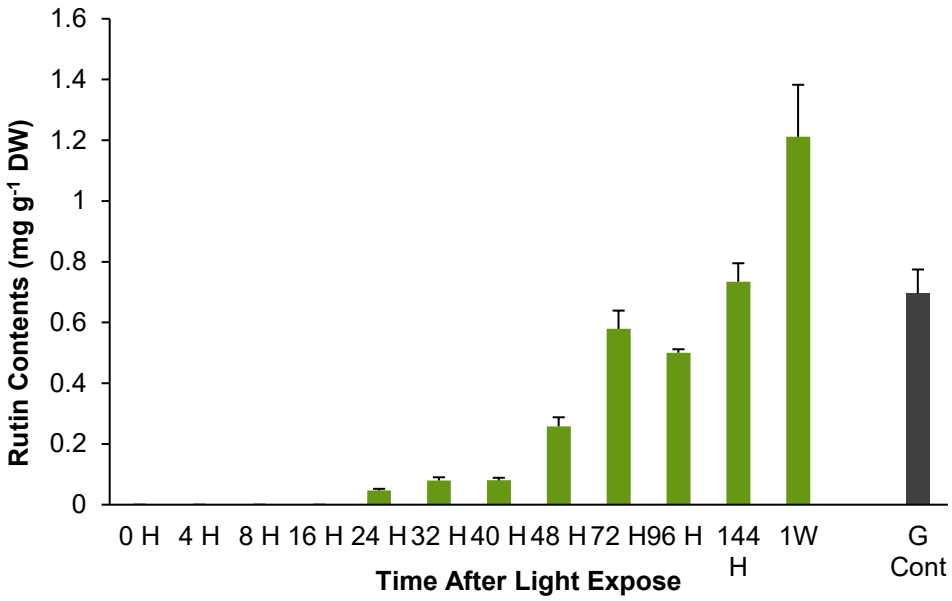

Fig.32 Rutin content on asparagus spears after light exposure to the natural light during winter fusekomi forcing culture. 0 h: white spear after open (AO); 4 h: 4 hours AO; 8h: 8 hours AO; 16 h: 16 hours AO; 24 h: 24 hours AO; 32 h: 32 hours AO; 40 h: 40 hours AO; 48 h: 48 hours AO; 72 h: 72 hours AO; 96 h: 96 hours AO; 144 h: 144 hours AO; 1W: 1 week AO. Bars indicated SD (*n*=3).

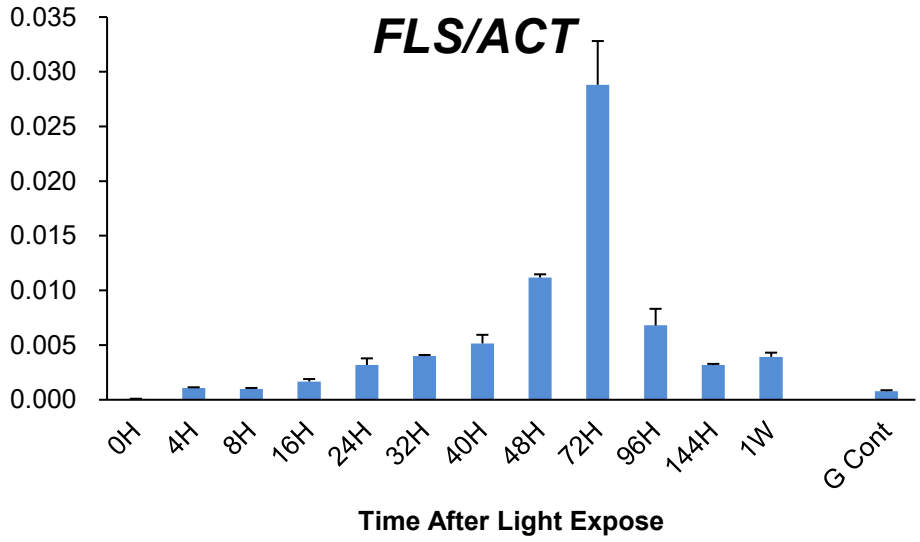

Fig.33 Gene expression of *Flavonol Synthase* (*FLS*) on asparagus spears after light exposure to the natural light during winter fusekomi forcing culture. 0 h: white spear after open (AO); 4 h: 4 hours AO; 8h: 8 hours AO; 16 h: 16 hours AO; 24 h: 24 hours AO; 32 h: 32 hours AO; 40 h: 40 hours AO; 48 h: 48 hours AO; 72 h: 72 hours AO; 96 h: 96 hours AO; 144 h: 144 hours AO; 1W: 1 week AO. Bars indicated SD (*n*=3).

Rutin was not detected on spears harvested at 0 h to 16 h after open. However, as the time exposure increases rutin contents gradually increase. Rutin increased sharply from 24 h and reach to highest at 1 W after open (Fig.32). The gene expressions of *flavonol synthase (FLS)* were gradually increased after light exposed; 4 h after open (light exposed) the expression were sharply increased to 72 h after open and then decreased as it goes to 1W (Fig.33). The coloration in the spears was found at the begining on the tip of some spears at 8 h after open and continued until the spears become greener in 1 W after open (Fig.34).

DISCUSSION

Previous studies in genes expression of flavonoids related genes in white and green spear, indicated that only the downstream gene, *FLS* showing as the light related genes while other genes shows similar on its relative gene expression. Study in the effects of light intensity on flavonoid related genes in the previous section also showed that no particular pattern of flavonoid related genes on their relative gene expression except *FLS.* The expression level of *FLS* was increased according to the number of lamps. This results indicating that *FLS* plays an important role to enhance rutin biosynthesis.

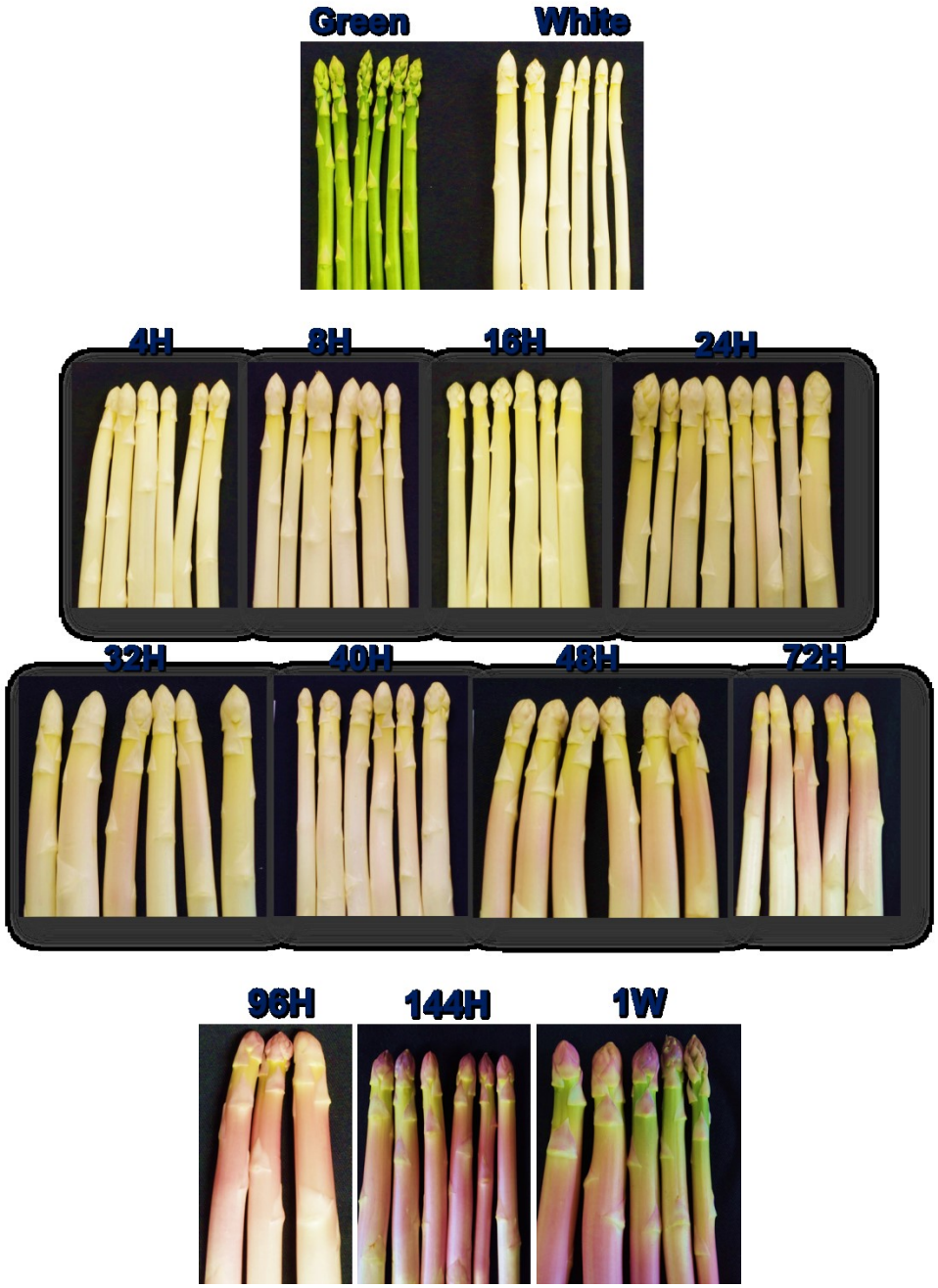

Fig.34 Coloration of the spears after light exposure to the natural light during winter fusekomi forcing culture. 0 h: white spear after open (AO); 4 h: 4 hours AO; 8h: 8 hours AO; 16 h: 16 hours AO; 24 h: 24 hours AO; 32 h: 32 hours AO; 40 h: 40 hours AO; 48 h: 48 hours AO; 72 h: 72 hours AO; 96 h: 96 hours AO; 144 h: 144 hours AO; 1W: 1 week AO. Green is the spears grown under natural light (without sun block).

In this section, rutin content was increased according to the length of light exposure; as the time of light expose increased, rutin content was gradually increased. Rutin was changing accordingly with the length of light exposure. Interestingly, spear color also changes accordingly as the time of light expose increased. As the colorations begin from white to green spears, rutin also increased. Rutin were not detected in 4 h, 8 h, 16 h after open, however it is start to increase on sharply 24 h after open and reach the max on 1 W. The coloration of the spears also happened in similar way. Interestingly, the gene expression of *FLS* began to increase sharply from 4 h after light exposed. These findings indicate that, in the molecular side, in short time of light exposure rutin regulation was stimulated and the synthesis started even before the pigmentation occurs; this may happen in purpose to protect the spears from light stress after open or light exposure began. Interestingly, the expression level of *FLS* reach to the max of its gene expression on 72 h after open and then decreased to 1W as the pigmentation began in the spears and almost near to green color. *FLS* was observed to have low expression level in the earliest pigmentation stage and decreased in the later stages (Ueyama et al., 2006; Noda et al., 2004). This may indicate that there might be the existence of some regulatory or transcription factors that controls *FLS* gene expression just after starting of light exposure to the unpigmented spears and after pigmentation begun. However, to

understand the mechanism of rutin regulation and its gene expression, further examination and studies of other flavonoid related genes and also regulator factors such as transcription factor are needed.

There are still few studies about the mechanism of rutin synthesis in asparagus spears. We clarified that the changes of rutin and *FLS* expression during the changing of light exposure time to asparagus white spears. Although further studies are needed, we found that *FLS* started to regulate even before the pigmentation began, this might explain the role *FLS* in asparagus spears.

CHAPTER 5

General Discussion

The experiments of this study were conducted in order to improve the quality of asparagus spears cultivated in the winter fusekomi forcing culture and to understand the mechanism of rutin biosynthesis and it is relationship with light. Supplemental lighting was introduced into fusekomi forcing culture system. Study about the effect of different numbers of supplemental lighting, length of time exposure and quality of supplemental lighting were conducted. We observed that supplemental lighting was effective to improve the quality (rutin contents and greener in color) of asparagus spear especially for the area that has low sunlight and/or in short day condition. According to the results of this studies conducted on 2012 to 2017; compared to the control plots, rutin content was significantly increased on the supplemental lighting plots (1 fluorescence lamp 16 hours and 24 hours; 2,3,4 fluorescence lamps 24 hours). Spear color was also significantly got better in the supplemental lighting plots than that of the control plots. Interestingly, according to the number of fluorescence lamps and length of the irradiation time, even among the supplemental plot, there was a significant difference in the color and also rutin content. Studies on summer-autumn cultivation (mother-fern cultivation) indicated the similar results, by improved light conditions, rutin were

increased and spear color became better (Maeda et al., 2010). In the study of the effects of light quality on rutin content, we found that, blue light for 24 h and FL1-24 h treatment has the same amount of rutin and has the highest rutin content against control. However, spears grown under blue light treatment has paler color compare to those grown under FL1-24 h treatment, suggesting that blue light was only efficient to enhance rutin (flavonoid biosynthesis) but not chlorophyll directly. It is thought that blue light/wavelength 400-450nm included in white florescent lamps responsible to enhance rutin in FL treatment. The same results also had been reported that buckwheat grown under blue light has higher rutin content than under natural light (Lee et al., 2001). Other studies were also indicating that flavonoid compounds were increased significantly when it exposed to blue light combine with UVB or white light. It was also suggested that irradiation with blue light can be used to induce the accumulation of phytochemicals that have positive impacts on human health via the induction of related genes (Ahn et al., 2015). These studies support our results that blue light specifically plays a role to stimulate rutin biosynthesis and might have relation with photoreceptor (Campbell et al., 1976; Suzuki et al., 1987; Hofmann et al., 2000, 2001, 2003). In contrast, UV-B causes severe damage to the spear. Spear become hard, malformed and the color become paler. This damage had leads to the low rutin content. It seems like the

dozes of UV-B were too much and causes stresses to the spears in this study. Former studies reported that UV-B alone without synergistically work with another light such as blue light and/or white lamps shows no effects (Ebizawa et al., 2008). The addition of UV-B with small amount of dozes and in some prior irradiation time is believed to work to enhance rutin (Goto et al., 2016). It is thought that light intensity or the dozes and irradiation time play an important role in this case beside the combination of several light qualities.

Rutin content tends to fluctuate according to the light environment; rutin is getting higher due to the quantity, intensity, and exposure time of supplemental lighting. Additionally, the effects of different light quality on asparagus spears suggested that blue light work specifically to increase rutin. These result suggested that rutin biosynthesis in asparagus spears was enhanced by supplemental lighting and blue light especially are found effectively enhanced rutin biosynthesis.

To understand the mechanism of how light enhance rutin regulation, gene expression analysis was conducted. White spears grown under dark condition and has been reported to contain saponin (protodiscin) not rutin (Maeda et al., 2012). According to our results, rutin only detected in green but not in white spears. By using the sample used in rutin analysis, analysis of flavonoid related genes expression was conducted.

The earliest genes of the flavonoid pathway in asparagus were expressed in unpigmented white spear almost similar with green spear. It is though that, the expression of earliest flavonoid gene in unpigmented white spear might involve in synthesis of other secondary metabolites (Boss et al., 1996). In contrast, the downstream or latest genes on the flavonoid pathway, especially *FLS* was significantly lower in white and the expression level were found almost drop to zero. In the study of the effect of light intensity to the flavonoid related gene expression, we also found that *FLS* expression level was increased in light treated plot; FL4 was the highest whereas low in white spears. *Flavonol synthase (FLS)* catalyzes the reaction from dihydroquercetin to flavonol (quercetin/ rutin) are the key enzyme in the rutin biosynthesis (Holton et al., 1993; Pelletier et al., 1999; Wisman et al., 1998; Stracke et al., 2007). Some other studies also indicated that *FLS* were affected by light (Ebizawa et al., 2008; Li et al., 2001). These reports supported our finding that *FLS* needed light and *FLS* is one of the key enzyme and/or light related genes in asparagus flavonoid biosynthesis pathway.

To understand relationship between rutin regulation and light more detail, first we made white asparagus and exposed the spear to the light until the spear become green (Fig.31). The samples were harvested at 0 h after open (white spears) to 1 week period (spears become greener). As the coloring begin from white to green, rutin were

gradually increased. In other words, rutin increasing and coloration has correlation. While the expression of *FLS* began to increase even before the coloration and rutin were detected. These findings indicate that, in the molecular side; synthesis of rutin is already beginning just after light exposure, even before the coloration occurs. This also suggested that *FLS* might play an important role as light related gene. Further examination and studies on other flavonoid related genes and regulator are needed to understand the whole mechanism of rutin synthesis in asparagus.

In conclusion, by introducing and/or improve the light conditions in the fusekomi forcing culture, spear quality are become better (rutin content increases and better color). These studies also indicating that this culture system (method) could be used by the farmers, because it is easy to conduct, in addition it is also has the secondary merit as heat source. Rutin biosynthesis was enhanced by light and/or in accordance with the light intensity, quantity and quality. *FLS* is one of the key enzymes that reacts to the present/absent of light and play an important role in the rutin regulation.

SUMMARY

In Japan, winter fusekomi forcing culture has been conducted to produce asparagus in winter. However, there are some problems such as the color of spears is pale, and rutin content is lower due to the low light intensity. To solve this problem, supplemental lighting was introduced to 'fusekomi' forcing system. We found that according to the number of lamps and irradiation time, rutin contents were getting higher and spear color also getting better. Blue light shows significant role to enhance rutin whereas UV-B seems to cause damage to the spears. In order to understand the relationship between light and flavonoid biosynthesis in asparagus, the amount of rutin content and its gene expression analysis were conducted. *FLS* were significantly higher in green comparing to white spears which do not contain rutin. *FLS* is thought to be one of key enzymes in rutin biosynthesis. The results of this study about the effect of light intensity, rutin was increased according to the number of lamps, so the expressions of *FLS*. The results of the study about time after light exposure to white spears; rutin were found increased according to the number of lamps and gradually increasing as the pigmentation began on the spears. *FLS* was begun to increase even before the discoloration and rutin were detected. This finding indicating that, in the molecular side, biosynthesis of rutin is already beginning just after light exposure and even before the coloration occurred.

Further studies of other flavonoid related genes and other regulator elements are needed to understand the whole mechanism of rutin synthesis in asparagus. In conclusion, by artificially introducing or improve the light conditions in the fusekomi forcing culture, spear color become better and rutin content were increases. *FLS* gene plays an important role to increase rutin. These studies are also indicate that this culture system (method) could be used by the farmers, because beside it is easy to conduct, it is also has the secondary merit as heat source. *FLS* is one of the key enzymes that react to the present/absent of light and play an important role in the rutin regulation.

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