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Seasonal Variation of the Major Allergen Fra a 1 in Strawberry Fruit

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Strawberry (*Fragaria* × *ananassa*) contains a major allergen, Fra a 1, which causes oral allergic syndrome. Fra a 1 is a PR-10 homolog that is regulated by environmental conditions. The allergenicity of fruit caused by Fra a 1 may depend on the genotype or growing conditions. We analyzed the *Fra a 1.01* transcript levels and Fra a 1.01 protein levels in strawberry fruits of several genotypes across all seasons. In the preliminary rough screening, we selected the line WH1 bearing white fruit and the red-fruited cultivar 'Akihime'. Under the same environmental conditions, there was no significant difference in Fra a 1.01 levels between the two cultivars over several months, suggesting that receptacle color was not indicative of allergenicity caused by Fra a 1.01. Fruits cultivated under the same environmental conditions should be used for comparisons of the allergenicity among genotypes. Both 'Akihime' and WH1 accumulated significantly higher levels of Fra a 1.01 protein in winter than in spring. We investigated the effects of irradiation and low temperature as environmental factors controlling the accumulation of Fra a 1.01 in winter. A shading treatment on fruit did not significantly affect Fra a 1.01 protein accumulation in strawberry fruits. Regarding variations over time, the Fra a 1.01 protein content was higher in fruits harvested at midnight in January than in those harvested at other times and in other months. These findings suggested that the Fra a 1.01 protein accumulates in response to environmental factors such as cold stress.

Key Words: *Fragaria* × *ananassa*, immunoblotting, oral allergic syndrome, pathogenesis-related, ripening.

Introduction

Strawberry (*Fragaria* × *ananassa*) is a popular fruit worldwide. In addition to its versatility as a fresh or processed food, it has become a popular food for human health because it contains bioavailable components such as phenolic compounds (Afrin et al., 2016; Giampieri et al., 2014; Kurze et al., 2018). However, strawberries also contain allergens that can cause oral allergic syndrome (OAS) in some people. The main OAS symptoms are tingling and itching as an IgE-mediated allergic reaction, and anaphylaxis in serious cases (Ma et al., 2003). The symptoms of OAS result from eating fresh fruit (Ebisawa et al., 2017). Among food OAS patients in the Kanto region, 13%–17% are sensitized to strawberry fruit (Maeda et al., 2010; Ono et al., 2007). If the OAS symptomatic population continues to rise, this could reduce strawberry consumption.

The major allergen in *F.* × *ananassa* is a member of the PR-10 subfamily, Fra a 1 (Hjernø et al., 2006; Karlsson et al., 2004), and that in *Fragaria vesca* is Fra v 1 (Hyun and Kim, 2011). The number of paralogs in the genome differ among genotypes; there are over 30 paralogs in *F.* × *ananassa* (Ishibashi et al., 2018), and 13 paralogs in *F. vesca* (Hyun and Kim, 2011). The Fra a 1.01 protein has been targeted as one of the major strawberry allergens (Ishibashi et al., 2018; Musidlowska-Persson et al., 2007).

Based on the experience of symptomatic patients, it has been suggested that Fra a 1 is related to white strawberry fruits (Hjernø et al., 2006). Transient RNA interference of *Fra a 1* reduced the red color of transgenic fruit (Muñoz et al., 2010), and the Fra a 1 protein has been shown to bind to specific flavonoids (Casañal

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et al., 2013). However, several studies found no relationship between Fra a 1 expression and fruit color (Franz-Oberdorf et al., 2017; Kaiser et al., 2016). To clarify this contradiction, it is essential to analyze Fra a 1 expression in white-fruited genotypes. There are several cultivars and varieties of strawberries in Japan that produce white and pale fruits. Analyses of these genotypes could be useful for selecting breeding materials with lower levels of the allergen.

In plants, PR-10 homologs function in development and defense (Fernandes et al., 2013; Liu and Ekramoddoullah, 2006). However, the specific functions of Fra a 1.01 related to biotic and abiotic stresses are unknown. Strawberry growing conditions vary among regions, with different types of equipment, and according to different cultivars (Caruso et al., 2011; Rahman et al., 2014). The conditions, cultivar, and ripening stage can all affect the concentrations of various components in strawberry fruits (Ariza et al., 2015; Ferreira et al., 2004).

Several studies have shown that the transcript levels of *Fra a 1* orthologs and the abundance of the encoded protein in other plant species are regulated by the growth conditions. For example, the transcript levels of *Ypr10* in the common bean (*Phaseolus vulgaris*) increased in response to a dark treatment and oxidative-stress (Walter et al., 1996), while the PmPR10 protein in the western white pine (*Pinus monticola*) accumulated in response to cold treatment and wounding stress (Liu et al., 2003). Various stresses have been shown to increase expression of PR-10 proteins in members of Rosaceae family, for example, Mal d 1 in apples (Wang et al., 2017). If the amount of Fra a 1.01 protein in strawberries is also affected by environmental factors, cultivation methods could be manipulated to regulate the accumulation of this allergen in strawberry fruits. Some reports have shown that the abundance of various components changes seasonally (Caruso et al., 2011; Vicente et al., 2014). The *Fra a 1.01* transcript levels and Fra a 1.01 protein levels in strawberries have also been suggested to vary seasonally. Thus, it is important to determine the background levels of Fra a 1.01 expression during the harvest season. In this study, we quantified the Fra a 1.01 protein in strawberry fruits of several genotypes over several months. We also investigated the effects of light and temperature on the levels of the Fra a 1.01 protein in strawberries.

Materials and Methods

Materials

In the first experiment, ripe strawberry fruits were obtained from three experimental farms in different districts. 'Akihime' and 'Wadahatsukoi' (*F.* × *ananassa*) were grown in Hokuto Yamanashi Prefecture (MIYOSHI AGRI-TECH CO., LTD.). Runner plants were settled on September 25, 2013 and harvested in early January, 2014. Two lines of 12112-01 and WH1, and a cultivar, 'Shirayukikomachi' (*F.* × *ananassa*), were grown in Kurume, Fukuoka Prefecture (Kyushu Okinawa Agricultural Research Center, NARO). Both 12112-01 and WH1 are breeding lines selected from seeds of a white fruited cultivar, 'Wadahatsukoi'. Runner plants were settled from late September, 2015 and harvested in late March, 2016. 'Tokun' (*F.* × *ananassa* × *nilgerrensis*; Noguchi and Yamada, 2014) was grown in Tsu, Mie Prefecture (Institute of Vegetable and Floriculture Science, NARO). Runner plants were settled from late September, 2014 and harvested in early February, 2015. The fruit peels appeared red ('Akihime'), white ('Wadahatsukoi', 12112-01, WH1, and 'Shirayukikomachi'), or pale yellowish-orange ('Tokun'). At each place, a forcing culture was conducted with soil hydroponics in a greenhouse. Each fruit was frozen in liquid nitrogen, ground into a powder at 2,000 rpm by a multi-bead shocker (Yasui Kikai, Osaka, Japan), and then stored at −80°C until analysis.

In the second experiment, 'Akihime' and WH1 were grown under the same conditions in a glass greenhouse at Kobe University (Kobe, Japan). In October 2017, two or three runner plants were cultivated in a plastic container filled with general culture soil and fertilized with Hyponex solution (Hyponex Japan, Osaka, Japan). From December 2017, a heating boiler was used to maintain the temperature above 5°C. A ventilation fan was used to keep the room temperature below 25°C. From January to May 2018, ripe fruits were harvested once a month (January 25, February 11, March 15, April 19, and May 4). We randomly chose a flower cluster from a plantlet of each cultivar in the same experimental period and harvested a fruit among second to fourth borne fruits from the flower cluster. Four fruits were used as replications for each designed treatment.

In the third experiment, for the shading treatment, unripe fruits were wrapped in aluminum foil from the green or white stage. The fruits were harvested at approximately 3–5 weeks after the confirmation of artificial pollination success by observing the achene formation on green receptacles. To analyze daily variations in Fra a 1 accumulation, ripe fruits ('Akihime') were harvested at 0:00, 6:00, 12:00, and 18:00 in January 11, March 19, and May 1, 2018. Each fruit was characterized, frozen in liquid nitrogen, ground, and then stored at −80°C until analysis.

Characterization of fruits

The strawberry fruits were characterized by measuring size, fresh weight, and color as described by Ishibashi et al. (2017). Fruit color was quantified by using a Handy Spectrophotometer NF 333 (NIPPON DENSHOKU, Tokyo, Japan) and scored by the L^*, a^* and b^* values.

A handy Brix-Acidity meter for strawberries (PAL-BX|ACID4; Atago, Tokyo, Japan) was used to measure the sugar concentration (Brix) and acidity (total acidity

as citric acid). Brix was determined for strawberry juice, while acidity was determined for a 1:50 dilution of strawberry juice.

Soluble proteins in fruit were quantified based on the Bradford method (Bradford, 1976). The fruit powder was mixed with modified extraction buffer (1:100 fruit powder:extraction buffer; Xie et al., 2010). A Protein Assay CBB Solution (5×) (Nacalai Tesque, Kyoto, Japan) was used for a colorimetric protein analysis with bovine serum albumin as the standard, according to the manufacturer's instructions.

Protein extraction and immunoblotting

Protein extraction and fluorometric determination were conducted as described by Ishibashi et al. (2018). Extracted proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 15% acrylamide gels and then blotted onto a polyvinylidene difluoride membrane by semi-dry transfer. To identify Fra a 1.01 protein, we used anti-Fra a 1.01e with 6 × His (1: 50,000 dilution) and alkaline phosphatase conjugated anti-guinea pig IgG (1: 3,000 dilution; ab102367, Abcam, Cambridge, UK). The peptides were detected with the appropriate antibodies and a BCIP–NBT solution kit for alkaline phosphatase staining (Nacalai Tesque) according to the instruction manual. Optical densities of the detected bands were quantified using Image J 1.50i software (National Institutes of Health, Bethesda, MD, USA). Separated proteins in 15% acrylamide gels were visualized by staining with Coomassie brilliant blue solution (Nacalai Tesque) (Fig. S1).

RNA extraction and real-time PCR

The RNA extraction, quantification, and reverse transcription methods were as described by Ishibashi et al. (2019). Real-time PCR was carried out in a LightCycler 480 (Roche Diagnostics, Basel, Switzerland) with Thunderbird SYBR qPCR Mix (Toyobo, Osaka, Japan). The PCR assay and thermal cycling conditions were as described by Ishibashi et al. (2019). The following gene-specific primers were designed for *Fra a 1.01* (forward: 5'-CACACCAAGGGAGATGTCG-3', reverse: 5'-GGGTGGTCCTTGAGGTATCC-3') and for elongation factor 1 α (*EF1α*) (forward: 5'-TGGATTT GAGGGTGACAACATGA-3', reverse: 5'-GTATACAT CCTGAAGTGGTAGACGGAGG-3'). The transcript level of *Fra a 1.01* was normalized against that of *EF1α*.

Statistical analyses

Statistical analyses were performed using JMP 13 (SAS Institute, Cary, NC, USA). We performed the Shapiro-Wilk test to confirm the normal distribution of data, and determined the significance of differences using parametric analyses. Data shown in Figures and Tables are the mean ± standard error of three to eight biological replicates.

Results and Discussion

Rough screening of cultivars

Several previous studies have suggested that the allergenicity of strawberries is related to fruit color, with white fruits being less allergenic than red ones. In Japan, cultivars bearing white fruits have been bred utilizing spontaneous mutations or by interspecific hybridization with wild species. Therefore, we roughly screened some white-fruited genotypes for a candidate to compare with 'Akihime', one of the major red-fruited cultivars in Japan. 'Akihime' was analyzed for Fra a 1.01 expression in our previous studies and used as a control (Futsuki et al., 2014; Ishibashi et al., 2018).

The *Fra a 1.01* gene and Fra a 1.01 protein were detected in all of the white-fruited genotypes, and their levels differed among genotypes (Fig. 1). The lowest expression levels of the *Fra a 1.01* gene and Fra a 1.01 protein were in the line WH1 and 'Shirayukikomachi'. The Fra a 1.01 protein levels were lower in 'Tokun' and

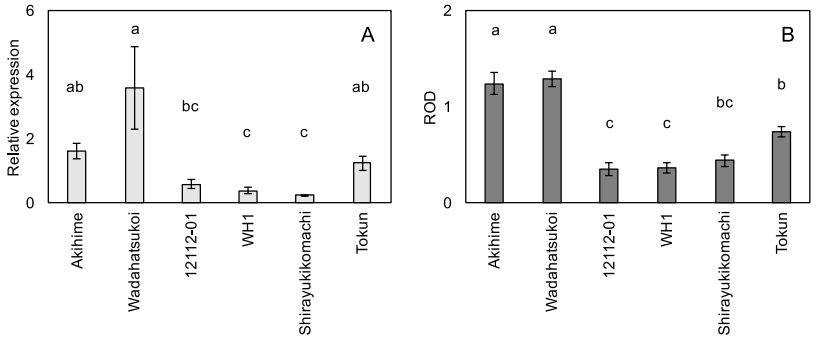

Fig. 1. Transcript levels of *Fra a 1.01* and concentrations of Fra a 1.01 protein in fruits of various strawberry genotypes. Fruits were harvested from 2014 to 2017 from plants grown under different cultivation conditions. Protein and RNA were extracted from each ripe fruit. Relative transcript levels of *Fra a 1.01* were detected by real-time PCR and normalized against that of elongation factor 1α (A). Fra a 1.01 protein was detected by immunoblotting with guinea pig antiserum including a polyclonal antibody raised against 6xHis-tagged recombinant Fra a 1.01e (B). Relative optical densities (ROD) were determined using Image J 1.50i software. Vertical bars show standard errors of four replicates. Different letters indicate significant differences (Tukey–Kramer's HSD test, $P < 0.05$).

the line 12112-01 than in 'Akihime'. The *Fra a 1.01* gene and Fra a 1.01 protein levels were not significantly different between 'Wadahatsukoi' and 'Akihime' when they were grown under the same conditions. From these results, we concluded that fruit color is not indicative of allergenicity related to Fra a 1.01. This finding was consistent with those of two previous studies on European varieties harvested in spring (Franz-Oberdorf et al., 2017; Kurze et al., 2018). One of the major reasons for no interaction between Fra a 1 expression and fruit color is the genetic variation in the flavonoid biosynthesis pathway in each white cultivar, that is, different mutations in an enzyme like phenylalanine ammonia-lyase (PAL) and/or dihydrokaempferol 4-reductase (DFR), a transcription factor like MYB, or a binding protein to metabolic intermediates like Fra a 1.

The six genotypes shown in Figure 1 were grown in three districts and in different seasons; therefore, we considered that the expression of Fra a 1.01 may be affected by environmental factors. This is consistent with the large variation in Fra a 1 levels detected in a previous outdoor cultivation experiment (coefficient of variation 43%; Alm et al., 2007). If environmental factors do affect Fra a 1.01 expression, then allergenicity should not be evaluated by comparing fruits from plants cultivated under different growth conditions. We selected WH1 as a line that produces a low level of Fra a 1.01 to clarify the effects of seasonal factors on Fra a 1.01 protein content.

Seasonal variation in Fra a 1 transcript levels and Fra a 1 protein levels

We grew the two genotypes under the same environmental conditions in Kobe, and compared their *Fra a 1.01* gene and protein expression levels. In both 'Akihime' and WH1, Fra a 1.01 protein accumulated to higher levels in winter (January and February) than in spring (Fig. 2). There was no significant difference in Fra a 1.01 protein concentrations between the two varieties over several months (Student's *t*-test, $P < 0.05$; data not shown). This was also the case when 'Tokun' was cultivated under the same conditions as 'Akihime' during another experiment conducted in other years (data not shown). These results clearly indicated that fruits from plants cultivated under the same environmental conditions should be used for comparisons of the allergenicity caused by Fra a 1.01 among genotypes.

The transcript level of *Fra a 1.01* in 'Akihime' was significantly higher in April than in winter, in contrast to the pattern of protein accumulation (Fig. 3). There was no correlation between *Fra a 1.01* transcript levels and Fra a 1.01 protein levels in 'Akihime' ($r^2 = 0.05$)

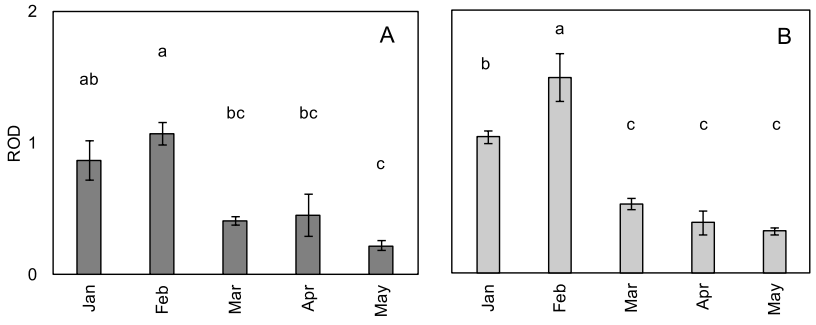

Fig. 2. Accumulation of Fra a 1.01 protein in strawberry fruits harvested from January to May 2018. Proteins were extracted from each ripe fruit that were identical to the sample in Table 1 (A: 'Akihime', B: WH1). Fra a 1.01 protein was detected by immunoblotting. Relative optical density (ROD) was determined as described in the Legend of Figure 1. Vertical bars show standard errors of four replicates. Different letters in each graph indicate significant differences among months (Tukey–Kramer's HSD test, $P < 0.05$).

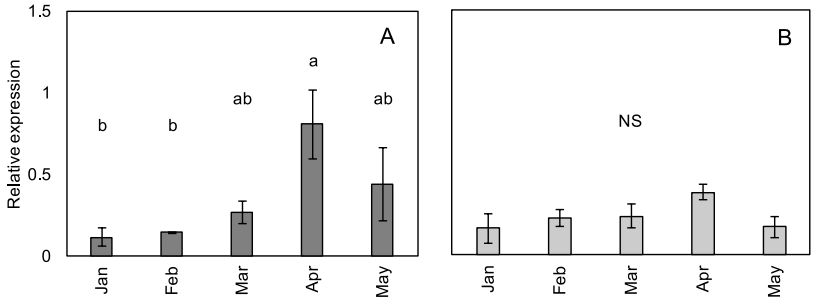

Fig. 3. Transcript levels of *Fra a 1.01* in strawberry fruits harvested from January to May 2018. RNAs were extracted from ripe fruit (A; 'Akihime': B; WH1) (see Fig. 2). Relative transcript levels of *Fra a 1.01* were detected by real-time PCR and normalized against that of elongation factor 1α (*EF1α*). Vertical bars show standard errors of four replicates. Different letters indicate significant differences (Tukey–Kramer's HSD test, $P < 0.05$). NS, not significant.

and WH1 ($r^2 = 0.06$). The lack of correlation may be indicative of a time lag between the half-lives of the mRNA and the protein (Schwanhäusser et al., 2011). Similarly, in the previous study as we analyzed the transcriptional and translational expressions in the same fruit, the difference in both expression levels may have reflected the time lag. In addition, post-transcriptional regulation or transportability through organs have been discussed as possible factors (Ishibashi et al., 2018). To clarify these points, more detailed studies will be needed regarding the earlier transcript levels or the protein identification in xylem sap. In this study, we focused on the protein level because the protein functions as a direct allergen.

We investigated fruit quality in two cultivars to determine whether any quality parameters were correlated with Fra a 1.01 expression. In forcing culture, the ripening period depends on the season, particularly the total effective temperature (Morishita and Honda, 1985). In this study, fruit ripening took 5 weeks in winter, longer than in spring (3–4 weeks). During these periods, the estimated integrated solar radiation in Kobe was 301 $MJ \cdot m^{-2}$ in January, 214 $MJ \cdot m^{-2}$ in February, 258 $MJ \cdot m^{-2}$ in March, 341 $MJ \cdot m^{-2}$ in April, and 292 $MJ \cdot m^{-2}$ in May (the New Energy and Industrial Technology Development Organization; http://app0.infoc.nedo.go.jp/index.html).

'Akihime' had a red-colored receptacle with a long cone form. The brightness (L^* value) and fresh weight of the fruits were significantly higher in winter than in other seasons (Table 1). There was no significant difference in redness (a^* value) and fruit length among months. WH1 produced a white-colored receptacle with a slightly oval form. The L^* value of WH1 fruits in winter was significantly different from that in May (Table 1). The sugar concentration (Brix) in the fruits of each cultivar was significantly higher in winter than in spring (Fig. S2). The soluble protein contents in 'Akihime' did not differ among seasons, so the optical density of Fra a 1.01 protein in the immunoblotting analyses reflected the total content of Fra a 1.01 protein in each fruit (Fig. 2). The fruit characteristics other than color were not significantly different between 'Akihime' and WH1 (data not shown). Seasonal variations were detected in fruit L^* value, fresh weight, and Brix (Table 1; Fig. S2). The Brix value was positively correlated with Fra a 1.01 protein content ($r^2 = 0.68$). It is possible that sugar synthesis and Fra a 1.01 expression are regulated by the same environmental factor, such as low temperature, as discussed later.

Some studies have described annual changes in Fra a 1 protein levels in strawberries cultivated in Europe. In Italy, no clear trend was observed, with only some genotypes having red colored fruit that exhibited an annual change (Tulipani et al., 2011). The pattern of change was either up- or down-regulation in each genotype. In Germany, there was no significant annual change in Fra a 1 protein levels among fresh fruits of a red cultivar (Kurze et al., 2018). To our knowledge, this is the first report that Fra a 1 concentrations in strawberries differ during the harvest season and among months. There are four distinct seasons in Japan, and the temperature fluctuates considerably among them. The daylight hours are longer and the air temperature is higher in spring than in winter (Fig. S3). Therefore, we determined the effects of various environmental factors on Fra a 1.01 protein accumulation in winter.

Effects of shading on Fra a 1 accumulation

We determined the effect of sunlight on both red- and

Table 1. General characteristics of strawberry fruits (*Fragaria* × *ananassa*) harvested in 2018.

Harvest month[z]	Fruit color			Longitudinal diameter (mm)	Transverse diameter (mm)	Fresh weight (g)
	L^*[y]	a^*[y]	b^*[y]			
'Akihime'						
Jan	42.4±1.1 a[x]	37.3±1.2 a	30.0±2.2 a	42.2±1.8 a	28.6±2.5 a	14.0±1.4 a
Feb	40.5±0.5 ab	38.9±0.7 a	30.3±1.6 a	37.5±0.7 a	26.8±0.6 a	10.5±0.6 ab
Mar	35.9±0.9 bc	37.2±2.0 a	26.6±1.9 a	35.5±1.1 a	24.5±0.8 a	8.4±0.7 b
Apr	33.1±0.5 c	37.7±1.9 a	25.2±1.1 a	40.9±2.7 a	26.0±2.4 a	10.1±1.8 ab
May	37.1±2.6 abc	35.8±1.4 a	24.2±2.0 a	35.0±2.1 a	24.6±1.3 a	7.4±1.0 b
WH1						
Jan	72.8±0.7 a	1.0±1.0 a	13.5±0.9 a	37.6±1.8 a	33.5±1.3 a	14.4±1.5 a
Feb	73.8±0.7 a	4.1±1.1 a	16.8±0.9 a	33.8±1.4 a	29.4±1.2 a	10.4±0.6 a
Mar	69.5±1.9 ab	4.5±3.3 a	14.4±2.7 a	37.8±2.6 a	33.1±1.8 a	14.3±1.8 a
Apr	67.1±0.8 bc	7.9±3.3 a	12.7±0.8 a	33.3±2.7 a	29.5±1.5 a	10.7±1.7 a
May	64.3±0.9 c	9.9±3.0 a	15.0±1.9 a	33.2±1.1 a	29.4±1.4 a	10.4±1.1 a

[z] Fruits were harvested at ripe stage approximately 3–5 weeks after fertilization. Data are mean ± standard error of four independent fruits.

[y] L^* meant the lightness (black [0] and white [100]), a^* meant difference of color between red (positive values) and green (negative values), and b^* meant difference of color between yellow (positive values) and blue (negative values).

[x] Different letters following values indicate significant differences among harvest months for each cultivar (Tukey–Kramer's HSD test, $P < 0.05$).

white-colored fruits by subjecting them to a shading treatment. The unripe fruits at the green or white stage were wrapped with aluminum foil to block the light. In 'Akihime', fruits that were shaded at the green stage showed inhibition of coloration and growth (Table 2). The Fra a 1.01 protein content was not significantly different among the control and shaded fruits (Fig. 4A). The *Fra a 1.01* transcript levels were significantly higher at the green stage (Fig. 5A). In the previous research, the transcript level of *Fra a 1.01* was correlated with maturing stage; the younger fruits expressed a higher amount of mRNA (Ishibashi et al., 2017; Petriccione et al., 2017). Meanwhile, the Fra a 1.01 protein accumulation level was constant during the ripening stages (Ishibashi et al., 2017). This tendency was consistent with the results of unshaded fruits at unripe stages (Figs. 4 and 5). This may have been related to the delay in ripening due to the shading treatment, rather than

Table 2. Effects of shading on strawberry fruits (*Fragaria* × *ananassa*).

Shading stage[z]	Fruit color			Longitudinal diameter (mm)	Transverse diameter (mm)	Fresh weight (g)
	L^*[y]	a^*[y]	b^*[y]			
'Akihime'						
Control	34.5±0.7 b[x]	37.5±1.3 a	25.9±1.0 a	38.2±1.7 a	25.3±1.2 a	9.2±1.0 a
Green	51.7±3.0 a	14.9±6.1 b	22.6±3.8 a	29.1±1.8 b	21.4±1.6 a	5.8±1.0 b
White	41.7±4.3 ab	18.4±5.9 b	20.7±1.7 a	31.7±1.3 b	22.3±0.8 a	7.1±0.7 ab
WH1						
Control	66.7±2.2 a	7.3±3.0 a	14.2±1.7 a	36.9±2.1 a	34.0±1.3 a	14.2±1.2 a
Green	65.9±2.0 a	−8.3±7.6 a	8.6±2.8 a	31.3±1.7 a	28.2±1.4 b	10.5±1.7 a

[z] Fruits were wrapped in aluminum foil at the green or white stage either 1–2 or 2–3 weeks after achene formation began, respectively. Each fruit was harvested at the mature stage, 4 weeks after the achene formation began during spring in 2018. Data are mean ± standard error of 4–8 independent fruits.

[y] Color data were determined as described in Table 1.

[x] Different letters following values indicate significant differences among different shading stages for each cultivar (Tukey–Kramer's HSD test, $P < 0.05$).

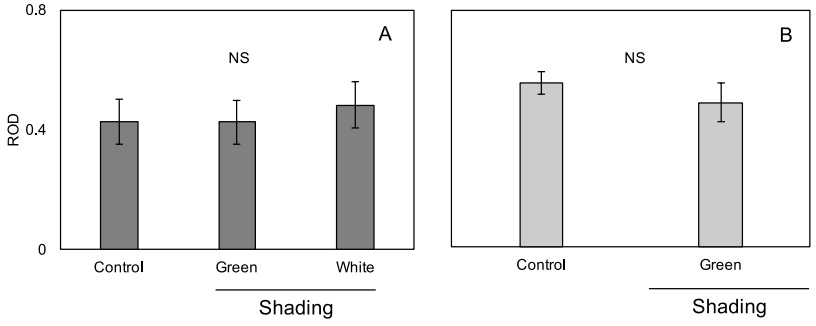

Fig. 4. Effect of shading on accumulation of Fra a 1.01 protein in strawberry fruit. Proteins were extracted from fruits in treatments shown in Table 2 (A; 'Akihime': B; WH1). Fra a 1.01 was detected by immunoblotting as described in the Legend of Figure 1. Vertical bars show standard errors of 4–8 replicates. Significant differences were not detected among treatments (Tukey–Kramer's HSD test, $P < 0.05$).

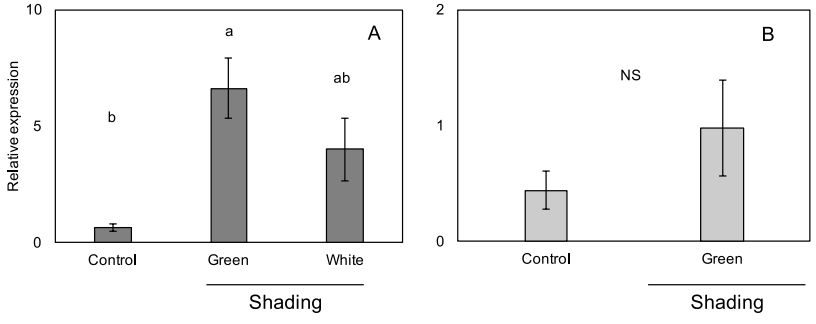

Fig. 5. Effect of shading on transcript levels of *Fra a 1.01* in strawberry fruits. RNAs were extracted from ripe fruit (A; 'Akihime': B; WH1) (see Fig. 4). Relative transcript levels of *Fra a 1.01* were detected as described in the Legend of Figure 3. Vertical bars show standard errors of 4–8 replicates. Different letters indicate significant differences (Tukey–Kramer's HSD test, $P < 0.05$). NS, not significant.

photoresponsivity. In WH1, neither the fruit color nor the expression of the *Fra a 1.01* gene and the Fra a 1.01 protein was significantly different between the shaded fruit and control fruit (Table 2; Figs. 4B and 5B).

In the strawberry growing district, the day length increased from January to May, but there was no statistically significant difference in monthly average daylight hours (Fig. S3). Estimated amounts of solar radiation in Kobe did not make much difference as mentioned in the preceding section. Therefore, solar radiation flux probably does not regulate Fra a 1.01 expression. In a previous study, UV-C radiation induced the accumulation of anthocyanin and Fra a 1 protein (Severo et al., 2015). Treatment with short wavelengths like UV-C radiation has been used to increase antifungal activity and antioxidant capacity (Erkan et al., 2008; Pombo et al., 2011), but it is also a stress factor that causes side effects. There may be an effect of different wavelengths beyond the solar spectra reaching the earth's surface. Further research is required to explore this topic.

Effects of low temperature on Fra a 1 expression

Air temperature is one of the most important seasonal factors affecting plants. The Fra a 1.01 protein content in strawberries was higher in winter than in spring, so we evaluated the effects of low temperature on Fra a 1.01 protein content. We analyzed fruits harvested from morning to midnight, and found that Fra a 1.01 accumulated to higher levels in fruits harvested at midnight than in those harvested at other times in January (Fig. 6). In that month, the temperature dropped below 5°C from midnight to dawn. In March and May, there was no significant difference in Fra a 1.01 accumulation among fruits harvested at different times of the day, and the temperature rose throughout the day above 15°C (Fig. 6). PmPR10 protein in *Pinus monticola* was induced by cold-hardening at 5°C (Liu et al., 2003). The temperature inducing the *DREB1* genes, the master switches for cold-responsive gene expressions, was mostly limited to 4–10°C in *Arabidopsis thaliana* (Kidokoro et al., 2017). We assumed that plants could recognize a low temperature stimulus as less than 10°C. Similar relations were found in rough screening (Fig. 1). The average air temperature during cultivation in each area was 4.4°C at Yamanashi ('Akihime' and 'Wadahatsukoi'), 5.7°C at Mie ('Tokun'), and 11.1°C at Kurume (12112-01, WH1 and 'Shirayukikomachi'). The cooler it became, the more Fra a 1.01 tended to accumulate.

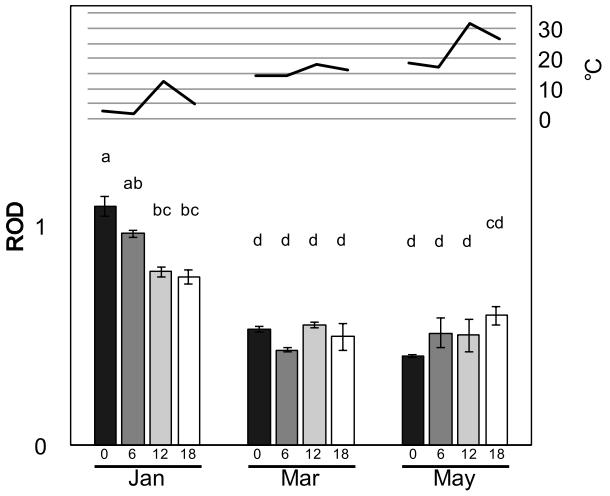

Fig. 6. Accumulation of Fra a 1.01 protein in strawberry fruits ('Akihime') during harvest periods. Bar charts show relative optical density (ROD) of Fra a 1.01 protein. Line graphs show mean air temperature in the greenhouse when each fruit was harvested. Proteins were extracted from each ripe fruit harvested at 0:00, 6:00, 12:00, and 18:00 from January to May 2018. Fra a 1.01 was detected and quantified as described in the Legend of Figure 1. Vertical bars show standard errors of three replicates. Different letters indicate significant differences (Tukey–Kramer's HSD test, $P < 0.05$).

In previous research on PR-10 proteins, several orthologs were found to be induced in winter and they functioned as antifreeze proteins. In the mulberry (*Morus bombycis*), WAP18 accumulated in the bark, xylem, bud, and cortical parenchyma, especially from October to March (Ukaji et al., 2004). In the white clover (*Trifolium repens*), TrVSP accumulated in roots and conferred chilling tolerance (Goulas et al., 2007). Previous studies have shown that the strawberry crown is sensitive to temperature, and is involved in regulating development or anthogenesis (Koehler et al., 2012; Lieten et al., 1995). In a proteomic analysis of cold tolerance, the Fra a 1.01 protein was found to be induced in the crown of the strawberry cultivar 'Jonsok' (Koehler et al., 2012). In *Cucurbita pepo*, MLPs, which are a subclass of PR-10 proteins, were found to be expressed in the roots and transported through the vascular bundle (Inui et al., 2013). The Fra a 1.01 protein has been shown to accumulate to particularly high levels in the roots and crown (Ishibashi et al., 2018). Therefore, we considered that the Fra a 1.01 protein may be regulated in response to cold stress, and accumulate in various parts of the plant.

In *F. vesca*, expressed sequence tags (ESTs) were developed from RNA extracted from strawberry organs exposed to abiotic stresses including low temperature (Rivarola et al., 2011). Futsuki et al. (2014) found 21 ESTs for a *Fra a 1.01* ortholog in the dataset for cold-stressed seedlings; the largest number of ESTs among all the *Fra a 1* orthologs. Several ESTs for products related to the carbohydrate metabolism pathway, such as sucrose synthase, were induced in the same cold treatment (Rivarola et al., 2011), suggesting that sugar synthesis and the accumulation of Fra a 1.01 protein may be regulated in a similar manner under cold stress. Previous studies have revealed that there are two putative low temperature responsive elements in the promoter region of gene07080, a *Fra a 1.01* ortholog in *F. vesca* (Alison Dunn et al., 1998; Ishibashi et al., 2018), and

one element in the *Fra a 1.01* promoter in *F.* × *ananassa* (unpublished data). Additionally, the transcript levels of *Fra a 1.01* were found to be high during the green stage and relatively low during the red stage (Ishibashi et al., 2017; Petriccione et al., 2017). We considered that the accumulation levels of Fra a 1.01 in winter may depend on regulation by cold stress at the early ripening stage. Otherwise, low temperature may be related to the post-transcriptional regulation, and/or the transportation of Fra a 1.01 protein.

Generally, June bearing strawberries are dormant during winter to resist cold stress (Kurokura et al., 2013; Sønsteby and Heide, 2006; Yamasaki, 2013). Both transcripts and translational products of PR-10 are implicated in cold tolerance and dormancy in woody plants as antifreeze proteins (Welling and Palva, 2006), suggesting that Fra a 1 induction by low temperature may be related to dormancy. In Japan, the deepest dormancy of strawberries is usually achieved in November and December. In this cultivation period, the tested cultivar 'Akihime' seemed unlikely to become deeply dormant as it had been shooting flower buds continuously after planting (data not shown). 'Akihime' is classified into cultivars with shallow dormancy, and analysis of the expression patterns with deeply dormant cultivars may reveal the relationship among Fra a 1, dormancy, and cold stress tolerance.

In conclusion, the Fra a 1.01 protein in several strawberry genotypes showed seasonal variations in its abundance. Its accumulation during winter may be caused by environmental factors such as low temperature. The promoter element that regulates the transcription of *Fra a 1.01* could be a useful target when attempting to breed less allergenic strawberry fruits. To modify the allergenicity of strawberries by controlling Fra a 1.01 accumulation during cultivation, we should determine the developmental stage and/or cumulative temperature that critically regulates the accumulation of the Fra a 1.01 protein.

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