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Review

Characteristics of major allergen Fra a 1 in cultivated strawberry

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Cultivated strawberry (*Fragaria* × *ananassa*) is a fruity vegetable of the Rosaease family and contains allergens which cause oral allergic syndrome (OAS). OAS is a type I allergy mediated by IgE, and as the symptoms appear in the oral cavity, hypoallergenic fruits are eagerly anticipated. The major allergen in strawberry OAS is Fra a 1—an ortholog of the birch pollen allergen Bet v 1. It is necessary to understand the characteristics of Fra a 1 *in planta* to properly regulate Fra a 1 accumulation and produce safe edible fruits. In this review, we summarize the Fra a 1 expression patterns in strawberries and this allergenicity in birch patients' IgE. Additionally, we describe the cultivar differences and environmental responses of Fra a 1 and discuss the proper regulation of Fra a 1 in order to produce hypoallergenic fruits.

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Introduction

Based on the definition of Ministry of Agriculture, Forestry and Fisheries in Japan, cultivated strawberry (*Fragaria* × *ananassa*) is a Rosaease fruity vegetable in horticultural crops. Ripe fruit is in high demand for both fresh and processed food, and breed branding is flourishing in each prefecture [1]. Nutritionally, strawberry fruits contain various ingredients good for tastes and human health [2], primary metabolites like sugars and organic acids, and second metabolites like polyphenols and aroma compounds [3–6]. In the meantime, strawberry has allergens, which cause oral allergic syndrome (OAS).

OAS is an IgE-mediated type I allergy; the symptoms appear in the oral cavity. Patients with OAS often manage their symptoms by taking internal medicines or avoiding causal fresh fruit [7,8]. Therefore, hypoallergenic fruits have been anticipated by breeding and/or cultivation control. In WHO/IUIS, three kinds of strawberry allergens have been identified: Fra a 1 in the pathogenesis-related protein 10 (PR-10) subfamily, Fra a 3 in the non-specific lipid transfer protein type 1, and Fra a 4 in profilin [9].

The major allergen is Fra a 1—an ortholog of birch pollen allergen Bet v 1 [10,11]. Bet v 1 family proteins are approximately 17–22 kDa, and the sequences are conserved in various species as PR-10 [12]. The patients who

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sensitize birch and other Rosaease allergen might also sensitize to Fra a 1 owing to cross-reactivity [13]. In Japan, approximately 13–17% of patients with food-induced OAS are sensitize to strawberries [14,15].

Accordingly, understanding the characteristics of Fra a 1 *in planta* is necessary to properly regulate the accumulation of Fra a 1 in order to produce safe edible fruits. In this review, we summarize the expression variations of Fra a 1 in strawberries (mainly *Fragaria* × *ananassa*) and this allergenicity in birch patients' IgE. In addition, we describe the cultivar differences and environ-mental responses of Fra a 1 and discuss the proper regulation of Fra a 1 for producing hypoallergenic fruits.

Expression patterns of *Fra a 1* paralogs in strawberry plants

Fra a 1 was first identified as the protein peptide before cloning the DNA sequence [10]. The peptide sequences were similar to the Bet v 1 family and were defined as a PR-10 group. In the DNA sequences, eight *Fra a 1.01* paralogs were first cloned from several cultivars [16], which was followed by the cloning of *Fra a 1.02* and *1.03* from "Camarosa" [17]. As *Fragaria* × *ananassa* genome has been sequenced from "Reiko" [18], 39 kinds of *Fra a 1* transcript sequences which encode 30 kinds of proteins were identified from the genome database [19]. These sequences are distributed in four clusters based on homology, and their expression patterns differ. In transcripts, *Fra a 1.02* paralogs are mainly expressed in fruit, especially at the receptacle [19]. The relative expression levels increase as the fruit matures [17,20]. However, *Fra a 1.01* paralogs are mainly expressed at the achene [19], and the expression levels decrease as the fruit matures [17,20,21]. In addition, the patterns of Fra a 1 protein accumulation in fruit differ from those of the transcripts. The protein accumulation is constant during ripening and richer in the receptacle than the achene, particularly in the Fra a 1.01 isoform [19,20]. We suggest that the allergenicity of strawberries should be evaluated according to the protein accumulation levels in each fruit owing to the low transcriptional-translational correlation in Fra a 1.01 expression.

Fra a 1 allergenicity has been evaluated by using several approaches *in vitro*. Some parts of recombinant Fra a 1.02 and Fra a 1.03 isoforms show the highest activation of basophils during the basophil activation test for birch pollen allergic patients [22]. However, in the immunoblotting test using IgE-specific birch, most IgE reacted with the recombinant Fra a 1.01 isoforms [19]. Both analyses showed large individual differences, even on the same test. In structural analyses, IgE responses on Bet v 1 family proteins are highly polyclonal [23,24], and Fra a 1.02 protein is reported to have several epitopes [25]. It is considered that allergen reactivity and/or these epitope sites vary widely based on individual and/or regional differences. As such, no universal allergen or epitope which responds to all patients has been found; hence, regulating the expression is desirable such that as many Fra a 1 peptides as possible are removed.

Furthermore, in the strawberry greenhouse, the patterns of sensitization to strawberry allergens could apply not only Fra a 1-induced OAS but also pollen allergy [26]. Several workers in strawberry greenhouse have work-related symptoms and sensitize strawberry pollen, but not all workers are necessarily sensitized to Fra a 1. Recently, another new allergen, gibberellin-regulated protein from strawberries was case reported [27]. We should be careful not to misinterpret these symptoms as OAS by Fra a 1.

Cultivar differences and genetic controls of Fra a 1

Breeding and variety selection is one approach in regulating Fra a 1 content in edible fruit. The possibility of linking allergenicity to fruit color has been reported. In Sweden, proteomics by MALDI-MS/MS showed that Fra a 1 content in white cultivars was lower than that in red [28]. Additionally, RNAi-mediated fruits toward *Fra a 1.01* and *Fra a 1.03* were partly whitened [17]. Fra a 1 proteins could bind to several flavonoids, so it was discussed whether the binding capacities of these flavonoids contributed to the regulation of secondary metabolic pathways in fruit [29]. However, white cultivar fruits in Germany and Japan contained Fra a 1 protein similar to that of red cultivar fruits when tested via ELISA and immunoblotting [30–32]. Especially in Japanese cultivars, the accumulation patterns of Fra a 1.01 in different colored ripe fruits were similar under the same environmental condition [32]. Only *Fra a 1.01a* was conserved in various cultivars in genotyping using microsatellite markers, regardless of receptacle colors [33]. Both genetically and environmentally, receptacle color could not be used as an indicator of allergenicity in the screening of cultivars. Therefore, comparing allergenicity in the same environment, regardless of the receptacle colors at present, is imperative. However, if another morphological feature linked to allergenicity were found, it would be a good indicator in the future.

In addition, as too many paralogs exist, as mentioned in the previous section, either gene transfer or genome editing should be a reasonable approach to reducing the total expression of *Fra a 1* genes. Down-regulation of *Fra a 1* has been reported by two methods for gene transfer—one is a transient system that produces fruits, implying that Fra a 1 is related to the anthocyanin biosynthesis pathway mentioned above [17]. The other is a stable transformation into the genome [34]. In the *Fra a 1.02*-silencing line, even though down-regulation of *glutathione-S-transferase* genes, transporter genes for *ABC* and *MATE* were found, no direct evidence was obtained regarding the relevancy between Fra a 1 and fruit color. Other functions of Fra a 1.02 in the defense response were indicated by the decreased expression of *WRKY* and *VQ23* and the diminished content of phytohormone (JA, SA, and IAA), which suggest that the Fra a 1 participates in essential plant physiological phenomena. These quality assess-ments or workarounds might be necessary in some plants.

Hypoallergic fruits should be obtained without the loss of necessary functions in plants. The selection of the specific promoter could be one of the solutions in controlling the gene expression in the desired tissues or developmental stages. Genome editing will also be effective by limiting the gene as a knockout target. It has an advantage in social acceptance by excluding the foreign gene after editing the target gene. Another consideration is that almost all cultivated strawberry species are reproduced vegetatively and genetically heterozygotes. T0 plant should be propagated vegetatively to avoid the segregation of other traits except for the target if the heterozygous cultivar was selected as an origin for transformation. Using the pure bred line will facilitate the genetic engineering of hypoallergic strawberries.

Environmental responses of Fra a 1

Regulation the Fra a 1 expression via environmental control is another approach. PR-10 proteins have defense mechanisms in response to biotic and abiotic stresses [35,36].

Gene and protein expression variations by various environ-mental responses have been reported as regards Fra a 1 (Table 1).

As a countermeasure against biotic stress, *Fra a 1* paralogs are induced in vegetative organs by *Verticillium dahlia* infection [37]. At this time, infected plants accumulate antifungal secondary metabolites and phytohormones.

Table 1 Expression variations of *Fra a 1* paralogs and Fra a 1 isoforms by environmental factors.

Environment	Gene or protein	Palalog or isoform	Expression pattern	Cultivar	Organ	Reference
Biotic stress						
Verticillium dahlia infection	gene	*others*	up	"Elsanta"	leaf, stem, root	[37]
Phytophthora cactorum infection	gene	undefined	up	*F. vesca* ("Hawaii 4")	root	[38]
Colletotrichum fructicola infection	gene	*1.01, 1.02,* and *others*	up	"Jiuxiang"	leaf	[39]
Abiotic stress						
UV-C radiation	protein	undefined	up	"Aromas"	fruit (ripe)	[43]
drying processes	protein	1.02	up	"Asia"	fruit (ripe)	[31]
chitosan coating	gene	*1.01a*	up	"Candonga", "Jonica", "Sabrina"	fruit (green, pink, red)	[44]
shading	gene	*1.01*	up	"Akihime"	fruit (from green or white)	[32]
	protein	1.01	n.s.*			
detaching achenes and pasting synthetic auxin	protein	1.01 and 1.02	up	"Akihime"	fruit (green, white, red)	[20]
wounding by surgical knife	protein	1.01	n.s.	"Akihime"	fruit (from green)	this article
cultivating in high-temperature	gene	*1.01*	up	"Redlands Hope", "Festival"	leaf	[47]
cultivating in low-temperature	gene	*1.01*	up	"Akihime"	crown, root	[48]
	protein	1.01	up	"Akihime", "Jonsok"	crown, fruit ('Akihime')	[48,50]
harvesting in low-temperature season	protein	1.01 and undefined	up	"Akihime", WH1, "Adria", "Svera"	fruit (green, pink, red, ripe)	[32,49]
chilled storage	protein	1.01	n.s.	"Akihime"	fruit (ripe)	[48]

* "n.s." indicates "not significant differences".

Fra a 1 orthologs in the wild strawberry root (*Fragaria vesca*) are induced in response to *Phytophthora cactorum* [38]. Several *Fra a 1* paralogs are induced in leaves by *Colletotrichum fructicola* infection [39]. Several PR-10 proteins possess ribonuclease (RNase) activity for these antifungus mechanisms [40,41]. In particular, phosphorylation status could affect RNase activity differently, although the degree depends on the plant species; pepper CaPR-10 increase activity [40], but cacao TcPR-10 has no effect [41]. Regarding Fra a 1, Fra a 1.06 protein, which belongs to Fra a 1.02 isoforms, has RNase activity [42]. Fra a 1.06 is not phosphorylated in the natural state. Other Fra a 1 isoforms are conferred with RNase activities via artificial dephosphorylation. These expressions and dephosphorylations may contribute to antifungus activities. However, the studies did not confirm the actual trends in protein accumulation and RNase activities when these fungi attacked strawberry plants. The function of Fra a 1 proteins against biotic stress should be identified in the future.

As a countermeasure against abiotic stress, Fra a 1 protein in fruit is induced by UV-C radiation and food processing [31,43]. These environmental conditions are often applied in improving the quality of post-harvest fruits. We should consider the side effects of post-harvest treatments and examine the conditions which will not affect allergenicity. In pre-harvest conditions, fruits with chitosan coatings induce *Fra a 1* transcript [44]. Shaded fruits from the early stage upregulate *Fra a 1.01* transcripts, but Fra a 1.01 accumulation does not change [32]. Sunlight could not significantly contribute to the regulation of Fra a 1 expression, and Fra a 1.01 expression might not be affected unless exposed to short and strong wavelengths, such as UV-C [43]. Additionally, several species induce PR-10 proteins via wound stress [45,46]. In strawberries, parthenocarpy-like fruits (detaching achenes and pasting synthetic auxin) induce Fra a 1.01 and 1.02 proteins [20]. Based on these, we compared the Fra a 1.01 accumulation in wounded fruits by using a surgical knife. Wound treatment alone several weeks ago did not affect on Fra a 1.01 expression (Table 1). More research is needed on short-term expressions, for example, immediately after fruit injury stress.

In addition, temperature stresses affect Fra a 1 expression. The transcript of *Fra a 1.01* paralog is upregulated in leaves in response to rising temperature in the high-temperature conditions [47]. Heat treatment of post-harvest strawberry extracts also slightly induces Fra a 1 content [31]. However, in low-temperature conditions, both *Fra a 1.01* transcript and protein in harvested fruit are not affected when exposed to cold conditions after harvest [48]. The effects of cold temperatures are noted before harvest. That is, Fra a 1.01 protein accumulates more in fruits harvested during winter and cold midnight in Japan [32]. A similar trend is observed in Italy, where Fra a 1 content is high when the temperature rise at the ripening stage is slow [49]. In the whole plants, *Fra a 1.01* transcript is first induced in the crown and root when the plants are exposed to low-temperature conditions during long-term cultivation [48]. Afterward, Fra a 1.01 protein accumulates in the crown and fruit. Similar patterns of Fra a 1.01 protein accumulation were confirmed at the crown exposed to cold for a month [50]. The temperature stress sensitivity of Fra a 1 might depend on the actual temperature zone, but it seems to be more sensitive before harvest. In cold stress, several PR-10 proteins are induced in winter as freezing resistance proteins [51,52]. Strawberry also might gain freeze tolerance by accumulating Fra a 1.01 protein at the crown as the temperature-sensing site and at the fruit as

the sink organ. Additionally, high- and low-temperature stresses could induce *Fra a 1.01* transcript expression, but protein accumulation patterns might not be the same, especially in post-harvest fruits. Other environmental stresses might regulate parts of *Fra a 1* paralogs and/or isoforms.

In conclusion, many *Fra a 1* paralogs are conserved in *Fragaria* × *ananassa*, which suggest various functions *in planta*. It is necessary to appropriately suppress the expression of as many paralogs as possible at once from the viewpoint of allergenicity, without losing the biotic and abiotic stress response functions of PR-10. In particular, we should be aware of the possibility that extreme stress treatments inadvertently induce the expression of Fra a 1, and we should avoid stresses as much as possible in pre- and post-harvest conditions.

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