



Analysis of major paralogs encoding the *Fra a 1* allergen based on their organ-specificity in *Fragaria × ananassa*

Misaki Ishibashi¹ · Takeshi Nabe² · Yoko Nitta³ · Hiroki Tsuruta⁴ · Miho Iduhara⁵ · Yuichi Uno¹ 

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Abstract

Key message *Fra a 1* protein in strawberry causes oral allergic syndrome. Over 39 *Fra a 1* paralogs have been identified in strawberry genome. *Fra a 1.01* is major accumulating protein in edible organs.

Abstract Strawberry fruits contain allergenic proteins that cause oral allergic syndrome. The hypothesized major allergen is *Fra a 1*, an ortholog of the birch pollen allergen protein Bet v 1. We organized *Fra a 1* genes and analyzed their localizations at the transcriptional and translational levels. In total, 15 new *Fra a 1* proteins were identified from the genomic database, increasing the total number of *Fra a 1* to 30 proteins encoded by 39 genes. *Fra a 1.02* was mostly expressed in receptacles, and *Fra a 1.01* in achenes, when analyzed by RNA sequencing. Immunoblotting showed that the *Fra a 1.01* protein was broadly accumulated in strawberry organs, while the *Fra a 1.02* protein was mostly expressed in receptacles. Recombinant *Fra a 1.01* strongly reacted with human IgE. The mRNA and protein expression levels of *Fra a 1* did not correlate, indicating the importance of protein levels when evaluating the abundance of allergens in strawberry. Based on the localizations, accumulation levels and reactivity to human IgE, we determined that *Fra a 1.01* was the most important allergen, followed by *Fra a 1.02*, and then other *Fra a 1* proteins. The information obtained here will be useful for selecting the target *Fra a 1* paralogs when breeding hypoallergenic strawberry.

Keywords Cultivated strawberry · IgE binding capacity · Oral allergic syndrome · Organ localization · Pathogenesis-related protein · RNA sequencing

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✉ Yuichi Uno
yuno@kobe-u.ac.jp
Misaki Ishibashi
151a302a@stu.kobe-u.ac.jp
Takeshi Nabe
t-nabe@pharm.setsunan.ac.jp
Yoko Nitta
nitta@fhw.oka-pu.ac.jp
Hiroki Tsuruta
tsuruta@kobe-u.ac.jp
Miho Iduhara
iduhara@biostir.com

Abbreviations

EF1α Elongation factor 1α
FPKM Fragments per kilobase of transcript per million mapped reads
OAS Oral allergic syndrome

¹ Department of Plant Resource Science, Graduate School of Agricultural Science, Kobe University, Rokko, Kobe, Hyogo 657-8501, Japan

² Department of Immunopharmacology, Faculty of Pharmaceutical Sciences, Setsunan University, Hirakata, Osaka 573-0101, Japan

³ Department of Nutritional Science, Faculty of Health and Welfare Science, Okayama Prefectural University, Soja, Okayama 719-1197, Japan

⁴ Research Unit for Future Creation and Innovation “Creative Dojo”, Graduate School of Engineering, Kobe University, Rokko, Kobe, Hyogo 657-8501, Japan

⁵ Biostir Inc., Annex Shin-Osaka 4A, 2-9-1 Miyahara, Yodogawa-ku, Osaka 532-0003, Japan

PTC	Post-transcriptional control
PR-10	Pathogenesis-related 10
RNA-seq	RNA sequencing

Introduction

Strawberry is a popular crop, with a unit price that is higher than those of other Rosaceae fruits (Gallai et al. 2009), and its demand has increased. Strawberry is rich in secondary metabolites, like polyphenols, which contribute to human health by increasing antioxidant activities (Giampieri et al. 2014). Their nutritional value has attracted not only the attention of consumers, but also breeding programs. However, strawberry fruit also contains allergens that cause oral allergic syndrome (OAS).

OAS symptoms caused by fruits or vegetables include itching, tingling, and swelling in the mouth or throat, and, in some cases, serious damage, like systemic symptoms and anaphylaxis (Ma et al. 2003). Unless anti-allergic drugs can be used, the main preventative strategy to cope with a fruit allergy is avoidance. However, this can limit the quality of life. Birch pollinosis patients are often sensitized to multiple allergens in Rosaceae fruits as a consequence of cross-reactivity with birch-specific IgE antibodies (Neudecker et al. 2001). Thus, there are more adult than child OAS patients because onset occurs after the establishment of a pollen allergy (Sicherer and Sampson 2010). Of the adult OAS patients in the Kanto region of Japan, 76% were sensitized to Rosaceae fruits, such as peach (31%), apple (22%), and strawberry (13%) (Ono et al. 2007). In Yokohama City, Japan, 16.6% of people diagnosed with OAS were sensitized to strawberry (Maeda et al. 2010). If the affected level of the population continues to rise, then it could lead to a slump in strawberry consumption. In addition, during production, farmers are exposed to plants, fruits, and pollen, and thus have a high allergic risk. Indeed, allergic reactions to strawberry in greenhouse workers has been reported (Patiwael et al. 2010).

To address the OAS problem associated with strawberry, preventative measures, such as the breeding of hypoallergenic fruits and reducing the expression of allergens through environmental control, are being undertaken. As a basic step towards these solutions, it is essential to comprehensively identify allergen genes, and to analyze their corresponding expressions. The first identified allergen was named as Fra a 1 (Hjærnø et al. 2006), and it is an ortholog of the birch pollen allergen protein Bet v 1. Fra a 1 is ~54% homologous to Bet v 1 (Hjærnø et al. 2006). These orthologs have similar three-dimensional structures, which could be the cause of the cross-reactivity (Seutter von Loetzen et al. 2012).

Other *Fra a 1* genes have been identified from cultivated species (*Fragaria* × *ananassa*) (Musidlowska-Persson et al. 2007; Muñoz et al. 2010; Amil-Ruiz et al. 2011; Franz-Oberdorf et al. 2016), and *Fra v* genes have been identified from the wild species *Fragaria vesca* (Hyun and Kim 2011). Musidlowska-Persson et al. (2007) obtained several *Fra a 1* gene sequences and determined that the Fra a 1 (Fra a 1.01) protein was the major allergen in strawberry. However, Franz-Oberdorf et al. (2016) conjectured that Fra a 1.02 and Fra a 1.03 were potent isoforms for OAS patients because of their abilities to cause higher basophil activation levels.

Using an expression analysis, Futsuki et al. (2014) reported that *Fra a 1.01* expression decreases during ripening. Muñoz et al. (2010) indicated that *Fra a 1.02* is highly expressed in ripe fruit. Both analyses targeted *Fra a 1* transcript levels using real-time PCR; however, the Fra a 1 protein has not been analyzed in depth.

The Fra a 1 protein belongs to the pathogenesis-related protein 10 (PR-10) family. PR-10 proteins are regulated by plant diseases, biotic/abiotic stresses, and plant hormones (Liu and Ekramoddoullah 2006). Allergen-like genes are highly expressed in response to crown rot *Phytophthora cactorum* in *F. vesca* (Toljamo et al. 2016), and the stress of UV-C treatments induces Fra a 1 protein accumulation in *F. × ananassa* (Severo et al. 2015). In addition to PR-10 functions, Fra a 1 is correlated with fruit pigmentation. Colorless (white) strawberry fruits have lower allergen levels than red strawberry fruits (Hjærnø et al. 2006; Alm et al. 2007). Muñoz et al. (2010) silenced *Fra a 1* genes in strawberry fruit by transient RNA interference, reducing the key enzyme genes of the flavonoid pathway and decreasing their red color, which indicated that Fra a 1 was related to pigment formation. Fra a 1.01e binds to quercetin-3-*O*-glucuronide and may act as a transporter of flavonoid intermediates (Casañal et al. 2013). In contrast, Kaiser et al. (2016) concluded that *Fra a 1.01* gene sequence is unrelated to fruit color because *fra a 1a2* was widely conserved among diploid strawberries, including white-colored varieties.

The number of gene sequences in the strawberry genome, the functional differentiation among paralogs, and which *Fra a 1* genes could be targets for allergen inhibition remain unknown. Recently, the strawberry genome was sequenced (*F. vesca*, (Shulaev et al. 2011); *F. × ananassa*, (Hirakawa et al. 2014)), and the resulting database provides new gene-related information. Next-generation sequencing technologies have been developed to simultaneously obtain expression and sequence data. In this study, we organized *Fra a 1* gene sequences by searching new gene identifications in the genomic database and by RNA sequencing (RNA-seq). Antibodies were raised against recombinant Fra a 1 proteins to detect the translational levels, and the organ-specific

expression levels of *Fra a 1* genes and proteins were analyzed. We also discuss the functional differentiation of *Fra a 1* paralogs based on their localizations and paralog's priorities in breeding.

Materials and methods

Plant materials

Strawberry (*Fragaria × ananassa* Duch., cultivar 'Akihime') was harvested and purchased directly from a working farm in Kobe City or sampled from a glass greenhouse at Kobe University, Kobe, Japan. Sets were purchased from a nursery in October 2014 and 2015. Three sets of plants were cultivated in plastic containers filled with a general medium. Each container held three plantlets, with three replications. Plants were watered and fertilized accordingly.

Ripe strawberry fruits at the red-colored stage were immediately frozen in liquid nitrogen. Achenes were separated from the receptacles of frozen fruits with a scalpel or tweezers. Petals, floral receptacles, sepals, peduncles, and anthers were removed from 5 to 10 flowers in full bloom. Leaf blades, leaf petioles, crowns and roots were separated from reproduced stocks. Birch pollen derived from *Betula pendula* was purchased as a medical sample for the allergic reaction. All of the samples were immediately frozen in liquid nitrogen, ground by a multi-bead shocker (Yasui Kikai, Osaka, Japan) at 2000 rpm, twice for 10 s, and stored at -80°C until use. A portion of each powdered sample was used for RNA and protein extractions.

Isolation and sequence analysis of *Fra a 1* genes

cDNA of each *Fra a 1* was amplified by RT-PCR to directly insert into the *NdeI* and *EcoRI* sites of a pColdII expression vector (Takara Bio, Kusatsu, Japan). PCR primers were designed for target genes and Factor Xa cleavage sites, ATCGAAGGTAGG (Ile-Glu-Gly-Arg), were added. Sequence information was obtained from Amil-Ruiz et al. (2011) to clone the coding region using primers for *Fra a 1.01* (forward: 5'-ATC ATC ATC ATC ATA ATC GAA GGT AGG ATG GGT GTT TAC ACT TAT GAA AAC GAG-3'; reverse: 5'-CGA CAA GCT TGA ATT TTA GTT GTA TTC GCT GGG GTG-3'), *Fra a 1.02* (forward: 5'-ATC ATC ATC ATC ATA ATC GAA GGT AGG ATG GGT GTG TTC ACT TAT GAA ACC-3'; reverse: 5'-CGA CAA GCT TGA ATT TTA ACA GTA TTC ATT AGG ATT GGC CAA G-3'), *Fra a 1.03a* (forward: 5'-ATC ATC ATC ATC ATA ATC GAA GGT AGG ATG GGT GTG TTC ACA TAC GAA TC-3'; reverse:

5'-CGA CAA GCT TGA ATT TTA GTT GTA TTC CTC AGG ATG GG-3'), and *Fra a 1.03b* (forward: 5'-ATC ATC ATC ATC ATA ATC GAA GGT AGG ATG GGT GTG TTC ACA TAC GAA TC-3'; reverse: 5'-CGA CAA GCT TGA ATT TTA GTT GTA TTC CTC GGG GTG G-3'). The RT-PCR products were gel-purified and cloned into a pColdII vector using an In-fusion HD cloning kit (Becton Dickinson, Franklin Lakes, NJ, USA) in accordance with the manufacturer's instructions. The vectors were transformed into *Escherichia coli* XL-1 blue. The cDNAs that failed in PCR-cloning were obtained by site-directed mutagenesis using a KOD Plus Mutagenesis Kit (Toyobo, Osaka, Japan) or gene synthesis using a GeneArt Strings DNA fragments (Life Technologies, Tokyo, Japan). In the site-directed mutagenesis, primers for *Fra a 1.01 b1* (forward: 5'-TAC ACT TAT GAA AAC GAG TTC ACC TCC GAT ATC CCA G-3'; reverse: 5'-AAC ACC CAT CCT ACC TTC GAT CAT ATG ATG ATG ATG-3') and for *Fra a 1.01 d* (forward: 5'-GTT ACA CTT ATG AAA ACG AGT TCA CCT CCG ATA TCC CAG-3'; reverse: 5'-CAC CCA TCC TAC CTT CGA TCA TAT GAT GAT GAT G-3') were used with *Fra a 1.01 a1* and *a2* cDNA, respectively, as templates. The genes for *Fra a 1.01a*, *Fra a 1.01c*, and *Fra a 1.03b* were selected for synthesis. The nucleotide sequences of both strands of the full-length cDNAs in the pColdII vector were determined using the dideoxy chain-termination method on an Applied Biosystems 3100 or 3130xi automated DNA sequencing system using the Prism Ready Reaction DyeDeoxy Terminator Cycle Sequencing kit 3.1 (Applied Biosystems Division, Perkin-Elmer, Foster City, CA, USA). Database searches were conducted using the National Center for Biotechnology Information network version of the BLAST algorithm with the strawberry sequence database, Strawberry GARDEN (<http://strawberry-garden.kazusa.or.jp>). Multiple sequence alignments were conducted using the CLC Main Workbench Version 7.7 sequence analysis programs (QIAGEN Aarhus A/S, Aarhus, Denmark).

Extraction, quantification, and qualification of RNA

RNA was extracted using the manual method for achenes and anthers, and the automated method for the other organs. Briefly, a Maxwell 16 Automated Purification system (Promega, Madison, WI, USA) with Fruit-mate for RNA Purification (Takara Bio) was used for the automated RNA extractions. The manual RNA extraction method was performed using Fruit-mate for RNA Purification and Sepasol RNA Super G (Nacalai Tesque, Kyoto, Japan), followed by a DNase I (Takara Bio) treatment and phenol–chloroform extraction. Then, an RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) was used according to the instruction manual. The resulting RNA samples were stored at -80°C .

Absorbance was determined with either a GeneQuant 1300 (GE Healthcare Ltd, Buckinghamshire, UK) or a Nanodrop1000 (Thermo Fisher Scientific, Waltham, MA, USA) spectrophotometer. The electropherograms, RNA integrity numbers, and 28S/18S ratios of the RNA samples for RNA-seq were examined using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

Quantitative real-time PCR

A ReverTra Ace qPCR RT kit (Toyobo) was used for the reverse transcription of 100 ng of total RNA according to the manufacturer's protocol. The cDNA solution was diluted by twice and stored at -20°C . Real-time PCR was carried out using a LightCycler Nano (Roche Diagnostics, Basel, Switzerland) with THUNDERBIRD SYBR qPCR Mix (Toyobo). Each PCR assay (total volume, 20 μL) contained 10 μL THUNDERBIRD SYBR qPCR Mix, 2 μL cDNA solution, and 10 μM specific primers. Specific primers were designed for *Fra a 1.01* and *Fra a 1.02* genes (*Fra a 1.01*-forward: 5'-CAC ACC AAG GGA GAT GTC G-3', *Fra a 1.01*-reverse: 5'-GGG TGG TCC TTG AGG TAT CC-3', *Fra a 1.02*-forward: 5'-ACA CCA AAG GTG ACG TGG A-3', *Fra a 1.02*-reverse: 5'-ATT AGG ATT GGC CAA GAG GTA G-3'), and for the elongation factor 1 α gene (*EF1 α*) as the control (forward: 5'-TGG ATT TGA GGG TGA CAA CAT GA-3', reverse: 5'-GTA TAC ATC CTG AAG TGG TAG ACG GAG G-3') (Amil-Ruiz et al. 2013). The thermal cycling conditions were as follows: 95 $^{\circ}\text{C}$ for 30 s, three-step amplification with 40 cycles of 95 $^{\circ}\text{C}$ for 15 s, 60 $^{\circ}\text{C}$ for 30 s, and 72 $^{\circ}\text{C}$ for 30 s. The amplification was immediately followed by pre-melt hold at 95 $^{\circ}\text{C}$ for 10 s, and melting at 60 $^{\circ}\text{C}$ to 97 $^{\circ}\text{C}$ at 0.1 $^{\circ}\text{C}/\text{s}$ to obtain a dissociation curve. All of the data were normalized against the level of *EF1 α* transcripts. Analyzing results were checked for efficiency (between 1.8 and 2.0), the slope of the standard curve (between -3.6 and -3.1), and only one melting peak.

RNA-seq analysis

Total RNA was obtained from the frozen powder of receptacles, achenes, and anthers. Each extracted total RNA was submitted to a custom order service (BGI Japan, Kobe, Japan) for cDNA library preparation and sequencing reactions. The cDNA libraries were sequenced on a HiSeq4000 platform (Illumina, San Diego, CA, USA) using paired-end chemistry with read lengths of 100 bp. Sequencing reads were filtered by SOAPnuke and mapped to the *Fragaria* $\times$ *ananassa* reference cDNA database (FAN_r1.1_cds; Hirakawa et al. 2014) by Bowtie. TopHat was used to determine exon expression.

Bacterial expression of recombinant Fra a 1 proteins

Each pColdII-Fra a 1 construct was transformed into BL21 (DE3) pLySs cells. Luria–Bertani medium (10 mL) supplemented with ampicillin (100 ng/mL) and isopropyl-D-thio-galactopyranoside (final concentration 1 mM) was inoculated with overnight cultures in accordance with the manufacturer's instructions. The cells were harvested by centrifugation, and the pellets were disrupted using the B-PER bacterial protein extraction reagent (Takara Bio) or using a sonicator. After centrifugation, supernatants containing His-Fra a 1 proteins were purified using a HIS Spintrap Kit (GE Healthcare) in accordance with the instructions.

Production of antisera to Fra a 1 proteins

Antiserum to recombinant Fra a 1.01e was produced by immunizing a guinea pig with a 1:1 (v:v) mixture of purified 6 $\times$ His-tagged Fra a 1.01e fusion protein (50 μg) and Freund's complete adjuvant. The guinea pig was given two booster injections at 4-week intervals. The serum was collected on d 42 and tested for the presence of anti-Fra a 1 antibodies by immunoblotting. Antiserum against Fra a 1.02 was ordered through a custom service (Eurofin Genomics, Tokyo, Japan). It was obtained by injecting rabbits with a synthetic partial peptide of Fra a 1.02 and adjuvant. Amino acid (aa) residues NH₂-LANPNEY + (Ahx)C-COOH at positions from 153 to 160, were selected from a specific region to generate a synthetic peptide. The serum was collected and tested as described above.

Protein extraction

We used a modified phenol extraction protocol to extract proteins (Faurobert et al. 2007). Soluble protein was extracted from 0.1 to 1.2 g of powdered strawberry samples using 5 mL of extraction buffer mix (0.5 M Tris-HCl pH 8.8, 0.1 M KCl, 0.7 M sucrose, 1.0% polyvinylpyrrolidone, 50 mM ethylenediaminetetraacetic acid, 1.0 mM phenylmethylsulfonyl fluoride in 2-propanol, 2.0% β -mercaptoethanol, and 0.1 $\times$ protease inhibitor). An equal volume of Tris-buffered phenol (pH 8.0) was added, and the sufficiently vortexed mixture was centrifuged at 3901g for 10 min at 4 $^{\circ}\text{C}$. The supernatants were stored in new tube, and then extracted again using an equal volume of fresh extraction buffer mix. Proteins were precipitated from the gathered supernatant using four volumes of 0.1 M ammonium acetate in cold MeOH overnight at -20°C . Centrifuged pellets were washed with the same solution three times. The final pellets were vacuum dried and dissolved in a buffer appropriate for polyacrylamide gel electrophoresis (PAGE). The concentration of each solution was measured

using a Qubit 2.0 fluorometer (Thermo Fisher Scientific). Samples were stored at less than -20°C until use.

SDS-PAGE

For SDS-PAGE, 1–2 μg of protein was dissolved in 20 μL of SDS-PAGE sample buffer (50 mM Tris-HCl pH 6.8, 0.25% SDS, and 10% glycerol) and separated by 15% acrylamide gel with prestained protein markers (Broad Range; Nacalai Tesque). After electrophoresis, the gel was stained with Coomassie Brilliant Blue Stain One Super (Nacalai Tesque) according to the kit instructions.

Immunoblotting

Recombinant or extracted proteins were separated by SDS-PAGE and blotted onto polyvinylidene difluoride membranes by semi-dry transfer. Peptides were detected with the appropriate antibodies and a BCIP-NBT solution kit for alkaline phosphatase staining (Nacalai Tesque). Anti-Fra a 1.01e with $6\times\text{His}$ (1:400 dilution) and alkaline phosphatase-conjugated anti-guinea pig IgG (1:3000 dilution; ab102367, Abcam, Cambridge, UK) were used to nonspecifically identify the Fra a 1 isoforms. The anti-Fra a 1.02 synthetic peptide (1:500 dilution) and alkaline phosphatase-conjugated anti-rabbit IgG (1:2000 dilution; S373B, Promega) were used to specifically identify Fra a 1.02. In competition assay, anti-Fra a 1.01e with $6\times\text{His}$ (1:25,000 dilution) was reacted with 1 or 10 μg of recombinant Fra a 1.01e or 1.02 proteins before blocking and antibody incubation of blotted PVDF membrane. Human sera or plasmas, which have specific IgEs to birch (1:100 dilution), and alkaline phosphatase-conjugated anti-human IgE (1:2500 dilution; A80-108AP, Bethyl Laboratories, Montgomery, AL, USA) were used to identify the cross-reactions with Fra a 1 isoforms. The product number (ellipsis) and manufacturer information for the human sera and plasmas are as follows: AE3998 (A) Biomedical Resources, Hatboro, PA, USA; C139363 (C), C-C Biotech Corporation, Valley Center, CA, USA; MC04 (M), International Enzymes, Fallbrook, CA, USA; R192138 (R), C-C Biotech Corporation; and VP14084 (V), Vital Products, Boynton Beach, FL, USA.

MS analysis to identify the primary protein structures

After SDS-PAGE and staining, target peptide bands were excised from the acrylamide gel to identify the primary structures. Peptide mass fingerprinting of the trypsin-digested peptides was performed at the Center for Supports to Research and Education Activities at Kobe University. For

the MS analysis, trypsin-digested peptides were separated by Paradigm MS2/HTS-PAL HPLC (Michrom BioResources Inc., Auburn, CA, USA) and analyzed on an LTQ-orbitrap Discovery (Thermo Fisher Scientific). For the MS data, m/z values were queried against the NCBI database or a local database to identify Fra a 1 sequences using the MASCOT algorithm (Matrix Science, Boston, MA, USA).

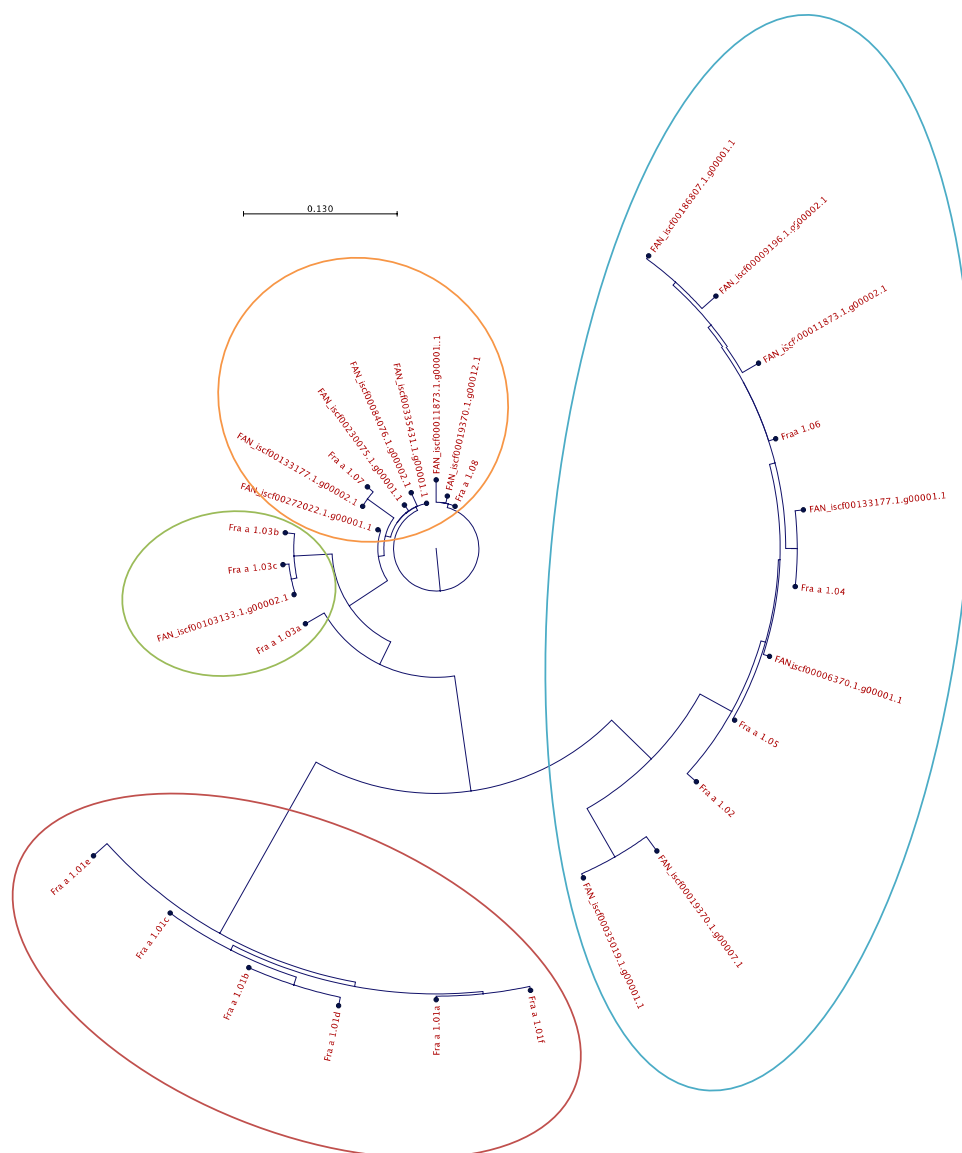
Statistical analyses

Statistical analyses were performed using JMP (SAS Institute, Cary, NC, USA) or R 3.2.1 software. Significant differences were determined by Student's *t* test, Welch's *t* test, or Wilcoxon's test. The data are means $\pm$ standard errors of more than four biological replicates or mixtures of 5–10 individual samples presented with three technical replicates.

Results

Organization of *Fra a 1* compared with newly identified gene sequences

In previous studies, 13 genes encoding Fra a 1 proteins were identified in *Fragaria* $\times$ *ananassa* (Musidlowska-Persson et al. 2007; Muñoz et al. 2010; Amil-Ruiz et al. 2011; Franz-Oberdorf et al. 2016). The branch numbers assigned for *Fra a 1* paralogs are 1, 2 and 3 or FaPR-10-1.1-1.5, 10-2, 10-3 and 10-4 (Table S1). We carried out PCR-based cloning to make constructs to produce recombinant proteins. In the process of cloning, two new *Fra a 1* gene sequences were identified and named based on sequence homology as *Fra a 1.01f* and *Fra a 1.03c* (Accession Nos. LC214965 and LC214966, respectively, Table S1). *Fra a 1.01f* shared 98–99% homology with *Fra a 1.01a-e* and 73–81% with other *Fra a 1* genes. *Fra a 1.03c* shared 98% with *Fra a 1.03b*, 90% to *Fra a 1.03a*, and 77–87% with other *Fra a 1* genes (Table S2). In addition, we searched the strawberry reference protein database version FAN_r1.1_pep (Strawberry GARDEN; <http://strawberry-garden.kazusa.or.jp>) for other Fra a 1 isoforms. In total, 21 Fra a 1 proteins were found in the database, including 5 redundant sequences reported in previous studies. For the Fra a 1.01 protein, only a partial sequence of Fra a 1.01e was found in the database (FAN_iscf00349897.1.g00001.1). A total of 30 Fra a 1 proteins encoded by 39 gene sequences were distinguished, and the deduced amino acid sequences were aligned to construct a phylogenetic tree (Fig. 1). Four clusters were formed, and each cluster included Fra a 1.01, 1.02, 1.03 and 1.07, respectively. The Fra a 1.02 cluster integrated Fra a 1.04, Fra a 1.05, and Fra a 1.06, having high homology levels of more than 96%. Similarly, the Fra a 1.07 cluster incorporated Fra a 1.08, with 95% homology.



Transcript analysis of *Fra a 1* by RNA-seq and real-time PCR

the achenes, and 57% (12/21) in the anthers. The itemized expression of each *Fra a 1* cluster is as follows: *Fra a 1.02* was greatest in the receptacles [fragments per kilobase of transcript per million mapped reads (FPKM)=2373], while *Fra a 1.01* was greatest in the achenes (FPKM=317). Other *Fra a 1* genes showed no or little transcription level, especially in fruit (the proportions of *Fra a 1.03–1.07* expression were 9.5% in the receptacles and 22.0% in the achene). Thus, we concluded that *Fra a 1.01* and *Fra a 1.02* were the major paralogs, which were targeted in further experiments.

The transcription levels were analyzed to understand the details of *Fra a 1.01* and *1.02* expression in edible parts of strawberry. The designed primer sets specifically recognized all nine *Fra a 1.01* sequences (*Fra a 1.01a1*, *Fra a 1.01a2*, *Fra a 1.01b1*, *Fra a 1.01b2*, *Fra a 1.01c*, *Fra a 1.01d*, *Fra a 1.01e1*, *Fra a 1.01e2*, and *Fra a 1.01f*) and five

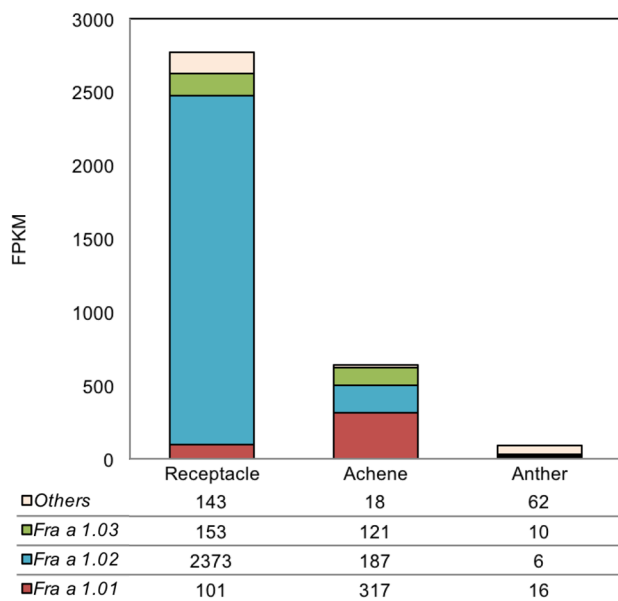


Fig. 2 Analyses of gene transcript levels in strawberry organs (*Fragaria* × *ananassa*) by RNA-seq. Using strawberry cultivar ‘Akihime’, RNA was extracted from receptacles (left) and achenes (center) of ripe fruits and anthers (right) of flowers in full bloom. Expressed transcript levels of *Fra a 1* were normalized as fragments per kilobase of transcript per million mapped reads (FPKM). Each bar includes the total FPKM for each *Fra a 1* gene cluster as shown in Fig. 1

of 11 *Fra a 1.02* sequences (*Fra a 1.02*, *Fra a 1.05*, FAN_iscf00006370.1.g00001.1, FAN_iscf00035019.1.g00001.1, FAN_iscf00019370.1.g00007.1). The quantitative real-time PCR revealed that the relative expression of *Fra a 1.01* in the achene was 46-fold greater than that in the receptacle, while that of *Fra a 1.02* in the achene was twofold lower (Fig. 3). These patterns of transcription levels were similar to the results from the RNA-seq analysis (Fig. 2).

To determine the role of *Fra a 1* in vegetative and reproductive organs, we investigated the expression levels of both *Fra a 1.01* and *Fra a 1.02* genes. *Fra a 1.01* expression was higher in petals and sepals among the tested organs (Fig. 3a). In particular, *Fra a 1.01* expression in red receptacles was the lowest of the reproductive organs. The *Fra a 1.02* expression pattern in petals was the highest of the tested organs (Fig. 3b). *Fra a 1.02* expressions in sepals and peduncles were similar to those in receptacles and achenes.

Accumulations of the *Fra a 1* protein in different organs

Translational products of *Fra a 1* genes were investigated to compare with the levels of the transcriptional products. First, we confirmed the specificity of the two kinds of antibodies raised against recombinant *Fra a 1.01e* and the synthetic partial *Fra a 1.02* peptide by immunoblotting (Fig. 4). In total, the cross-reactivity levels of ten recombinant *Fra a 1* proteins were tested to both antibodies. The antiserum to recombinant *Fra a 1.01e* recognized all of the *Fra a 1* proteins,

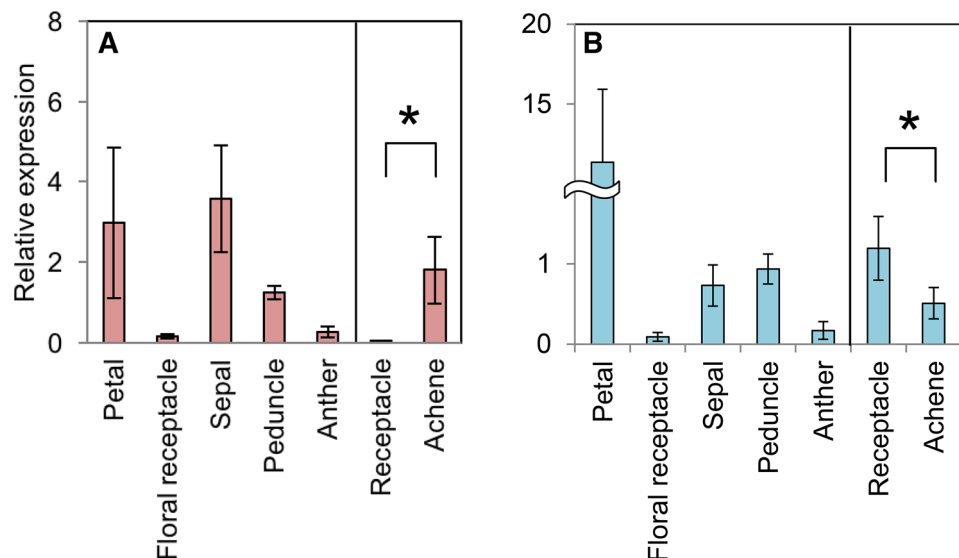


Fig. 3 Analyses of gene transcript levels in strawberry organs (*Fragaria* × *ananassa*) cultivar ‘Akihime’ by real-time PCR. RNA was extracted from receptacles and achenes of ripe fruits. The relative transcript levels of *Fra a 1.01* (a) and *Fra a 1.02* (b) were assessed by normalizing against that of the elongation factor 1 α gene (*EF1 α*).

Asterisks beside error bars indicate significant differences (a Wilcoxon’s test, $n=4$, $p<0.05$; b Welch’s t test, $n=3$, $p<0.05$). In case of other organs except fruits, RNA was extracted from the petals, floral receptacles, sepals, peduncles, and anthers of 5–10 flowers in full bloom from randomly selected individuals. (technical repetition = 3)

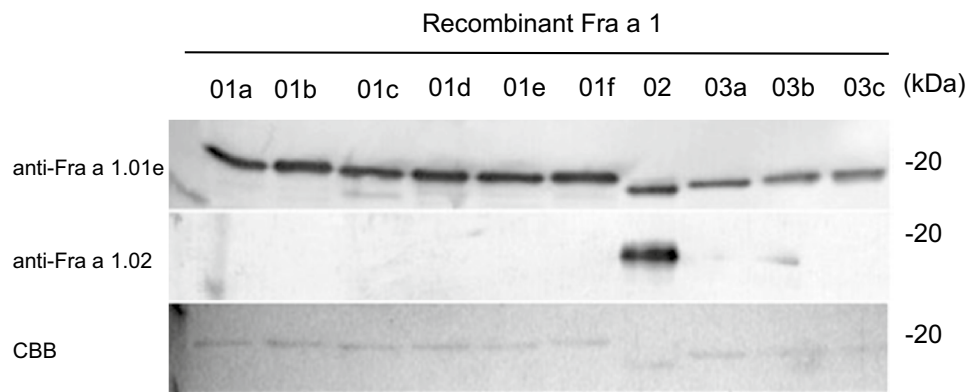


Fig. 4 Specificity of antibodies to recombinant Fra a 1 proteins. 6×His-tagged recombinant proteins were expressed in *Escherichia coli* BL21(DE3). Proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and detected by immunoblotting using guinea pig antiserum, including a

polyclonal antibody raised against 6×His-tagged recombinant Fra a 1.01e, or rabbit antiserum raised against a synthetic peptide [NH₂-LANPNEY+(Ahx)C-COOH] corresponding to a partial sequence of Fra a 1.02. Other sets of recombinant proteins were stained using Coomassie Brilliant Blue (CBB)

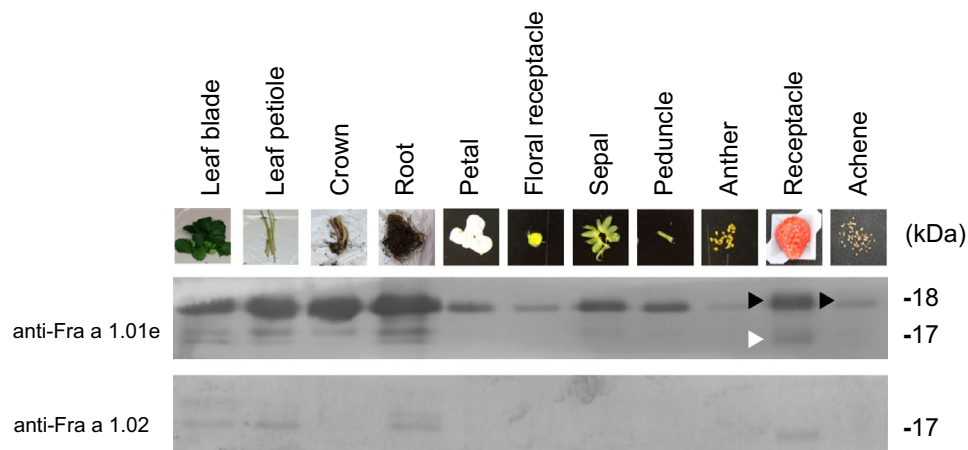


Fig. 5 Accumulations of Fra a 1.01 and Fra a 1.02 proteins in strawberry organs (*Fragaria × ananassa*) detected by immunoblotting. Using strawberry cultivar ‘Akihime’, proteins were extracted from various organs, that were sampled as shown in Fig. 3. Fra a 1 proteins were detected by guinea pig antiserum including a polyclonal antibody raised against 6×His-tagged recombinant Fra a 1.01e, or a rabbit antiserum including a antibody raised against a Fra a 1.02

peptide. Proteins were analyzed by SDS-PAGE with 15% acrylamide gels (Fig. S2). Three peptide bands from receptacle and achene were analyzed by MS analysis to identify the isoform. The high and low molecular bands from receptacle peptides were assigned as R1 (black arrowhead) and R2 (white arrowhead), respectively. From achene peptides, the band was indicated by black arrowhead and assigned as A1. Each number corresponds to band ID in Table 1

while the antiserum to the Fra a 1.02 peptide specifically recognized Fra a 1.02 only. Consequently, the former was used as a universal Fra a 1 antibody, and the latter was used as a specific Fra a 1.02 antibody (Fig. 5). The universality of the guinea pig antiserum to recombinant Fra a 1 was confirmed by competition and dose–response assays (Figs. S3 and S4). Fra a 1 allergens using the Fra a 1.01 antibody were detected in the receptacle and achene (Fig. 5). In the receptacle, two different bands appeared and were defined as R1 and R2 with high and low molecular weights, respectively. The R1 protein was expected for Fra a 1.01 based on its

molecular weight (18 kDa) and the R2 was expected for Fra a 1.02, 1.03 or 1.07 based on the molecular weight (17 kDa). To confirm this hypothesis, R1 and R2 were analyzed using an Orbitrap MS to identify the primary structure, together with A1, which is another protein from the achene (18 kDa; Fig. 5). R1 and A1 showed 56–66% sequence coverage of Fra a 1.01e and gene07080, which is an identical ortholog in *F. vesca*, respectively (Table 1). The sequence coverage of R2 was 61% of Fra a 1.02 and gene07065, which is an identical ortholog in *F. vesca*. The other sequence coverages of R2 were lower, at 15–42% (Table 1). The peptides matched

Table 1 Identification of Fra a 1 using trypsin-digested peptides derived from proteins differentially expressed in strawberry fruit (*Fragaria × ananassa*)

Band ID ^a	Organ ^b	Identical protein	Score	Mass (M_r)	Sequence coverage (%)
A1	Achene	07080	222	17,830	60
		Fra a 1.01e	234	17,812	56
R1	Receptacle	Fra a 1.01e	349	17,812	66
		07080	315	17,830	66
R2	Receptacle	07065	271	17,558	61
		Fra a 1.02	250	17,602	61
		04962	176	17,570	42
		07084	69	19,414	22
		Fra a 1.03c	43	17,644	15

Trypsin-digested peptides were introduced into an LTQ Orbitrap Discovery mass spectrometer and identified by peptide mass fingerprinting

^aEach band was detected by SDS-PAGE and guinea pig antisera, including a polyclonal antibody raised against 6×His-tagged recombinant Fra a 1.01e (Fig. 5)

^bProteins were extracted from the achenes and receptacles of ripe 'Akihime' strawberry fruits

^cm/z values, scores and nominal masses (M_r) were queried against the NCBI database or a local database to identify Fra a 1 sequences using the MASCOT algorithm (Matrix Science, Boston, MA, USA)

the specific sequence positions that were able to distinguish each Fra a 1 protein (Fig. 6 and S1). Based on these results, we distinguished Fra a 1.01 from other Fra a 1 isoforms using its molecular size when employing the universal Fra a 1 antibody in the following experiments.

The Fra a 1.01 protein accumulated in all organs with varied expression levels (Fig. 5). Vegetative organs (leaf, crown, and root) and receptacles had especially greater accumulation levels, while those in the achenes and anthers were lower. The Fra a 1.02 protein was detected only in leaves, roots, and receptacles. The Fra a 1 translational patterns did not always match the *Fra a 1* transcriptional levels. The transcription and translation of Fra a 1.01 and Fra a 1.02 showed no significant correlations between relative expression ratios and optical densities in examined organs (data not shown).

IgE-binding capacities of Fra a 1 proteins

To evaluate the binding patterns of the recombinant Fra a 1 proteins to the IgE antibody in human, immunoblotting was performed. At first, we investigated the IgE-binding properties of five individual serum or plasma samples, including IgE specific to birch, with recombinant Fra a 1 proteins. Two plasmas (human A and M) reacted with all of the recombinant Fra a 1, two (human C and V) sera only reacted with the recombinant Fra a 1.01 proteins, and human R did not react with any Fra a 1 proteins (Fig. 7). Human R might not have anti-Bet v 1 antibody cross-reacting with Fra a 1, or have other antibodies against minor birch allergen. The cross-reactivity to recombinant Fra a 1 proteins varied among the individual IgE samples; however, the IgE from most people reacted mainly with Fra a 1.01 proteins.

Second, we analyzed the accumulation of IgE-binding proteins in various strawberry organs as determined by three serum or plasma samples, which were selected based on their high reactivity levels to recombinant proteins (Fig. 8). Positive bands of ~18 and 17 kDa were detected by immunoblotting and were inferred to include Fra a 1.01 and other

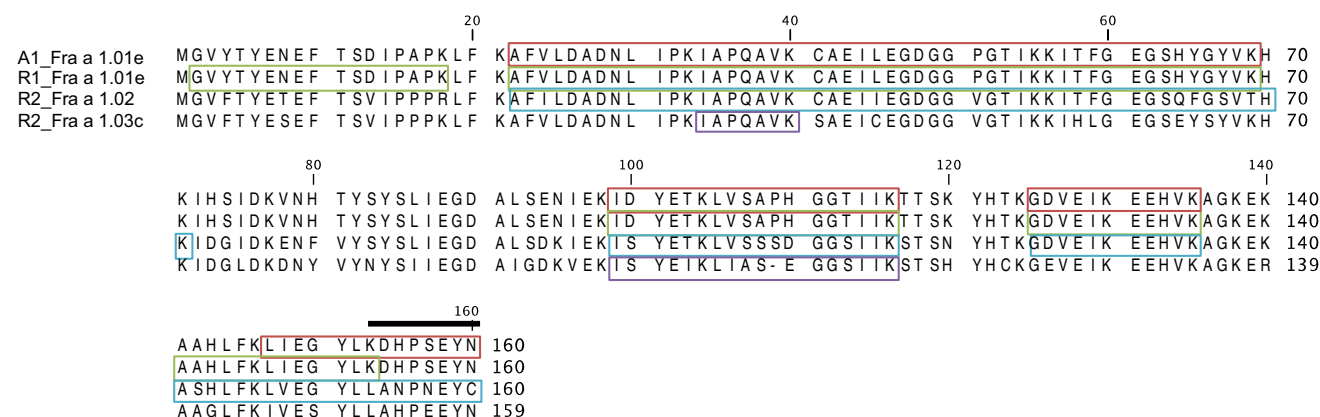
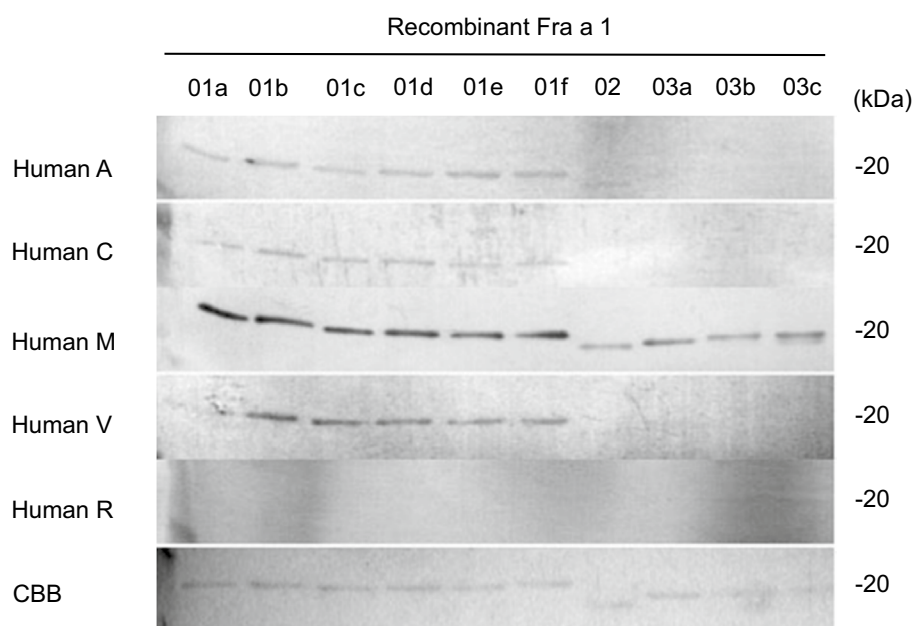


Fig. 6 Positions covered by the Fra a 1 peptide sequences in strawberry (*Fragaria × ananassa*) as determined by an Orbitrap mass spectrometer. Trypsin-digested peptides (Table 1) were introduced into an LTQ Orbitrap Discovery mass spectrometer and identified by peptide mass fingerprinting. Covered sequences are indicated by different

colored shades: red (A1, Fra a 1.01e), green (R1, Fra a 1.01e), blue (R2, Fra a 1.02), and purple (R2, Fra a 1.03c). The overlined amino acid residues (LANPNEYC) at positions 153 to 160 were used for a synthetic peptide to produce specific antibody against Fra a 1.02 (Fig. 4). (Color figure online)

Fig. 7 IgE-binding capacity to recombinant Fra a 1 proteins. 6×His-tagged recombinant proteins were expressed in *Escherichia coli* BL21 (DE3). Proteins were resolved by SDS-PAGE and immunoblotting using human sera or plasmas (Human A, C, M, V, and R), including IgE specific to birch. Alkaline phosphatase-conjugated goat anti-human IgE antibody was used as the secondary antibody. CBB staining of the gel is as same as in Fig. 4



Fra a 1 proteins, respectively. The accumulation levels of the proteins bound by IgE showed different patterns in each individual. The human A plasma bound only birch pollen protein, while the human C serum did not bind a protein, suggesting the detection limit with native protein. With human M plasma, 18-kDa bands were detected strongly in petal and weakly in sepal, peduncle, and red fruit, while 17-kDa bands were detected in all of the strawberry organs, as well as in birch pollen. Other strawberry allergens (nsLTP; 9 kDa, profilin; 12–15 kDa) were not detected in any organs.

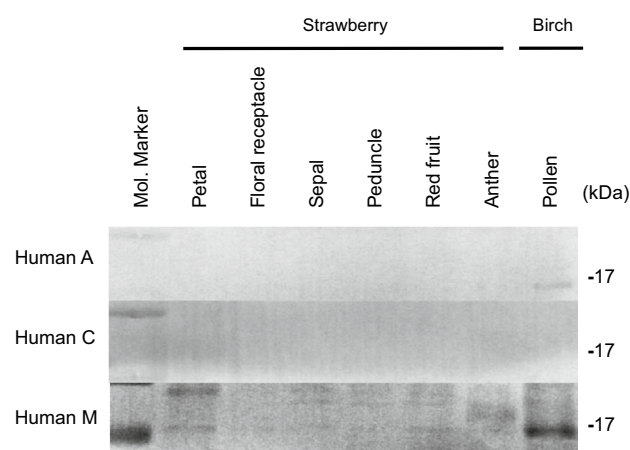


Fig. 8 IgE-binding capacities to strawberry proteins (*Fragaria* × *ananassa*) and birch pollen protein (*Betula pendula*). Proteins were extracted from strawberry organs as described in Fig. 5. The detection of proteins was carried out as described in Fig. 7

Discussion

Reclassification and expression analysis of *Fra a 1* in *F. × ananassa*

Fra a 1 is a major allergenic protein causing strawberry OAS, and it has many isoforms in cultivated strawberry (*F. × ananassa*; Musidlowska-Persson et al. 2007; Amil-Ruiz et al. 2011). In this study, we organized Fra a 1 and newly identified isoforms, and analyzed their localizations in organs to determine the paralog's priority in breeding hypoallergenic strawberry.

The whole-genome sequence of strawberry has revealed information regarding alleles and genes {*F. vesca* (Shulaev et al. 2011); *F. × ananassa* (Hirakawa et al. 2014)}. We found a total of 21 *Fra a 1* gene sequences in the *F. × ananassa* genome database (FAN_r1.1_cds; Hirakawa et al. 2014). Of these, 15 proteins were newly identified, indicating a significant contribution to the genomic database. The total number of *Fra a 1* gene sequences was 39, which is larger than the 16 found in woodland strawberry (*F. vesca*). However, cultivated strawberry is an octoploid species ($2n=8x=56$) with an estimated genome size of 1C=708–720 Mb, while *F. vesca* is diploid ($2n=2x=14$) with an estimated genome size of 209.8 Mb (Hirakawa et al. 2014). Since the number of *Fra a 1* genes may be estimated as ~54–64, more unidentified genes may exist. For example, only a partial sequence for *Fra a 1.01* (FAN_iscf00349897.1.g00001.1) was found in the database; nevertheless, many similar gene sequences were identified from a previous study (Musidlowska-Persson et al. 2007). Like *Fra a 1.01*, there were some partial Fra a 1 sequences in the

database. This is probably due to unassembled contigs, and/or unreadable sequences among scaffolds. The integration of information from the deep sequencing of RNA and gDNA will identify more *Fra a 1* genes in the future.

In total, 30 *Fra a 1* isoforms were used to construct the phylogenetic tree (Fig. 1). The four clusters contained 6 *Fra a 1.01*, 11 *Fra a 1.02*, 4 *Fra a 1.03* and 9 other *Fra a 1*. Based on this clustering, the expression levels of the *Fra a 1* were evaluated using the FPKM values from the RNA-seq (Fig. 2). The most expressed paralogous groups were that of *Fra a 1.02* in the receptacle, *Fra a 1.01* in the achene, and 'other *Fra a 1*' in the anther, which indicates functional differentiation in the *Fra a 1* family. In the strawberry's edible portion, additional values for *Fra a 1.01* and *Fra a 1.02* together accounted for 89% of the total expression in the receptacle and 78% in the achene. Thus, we concluded that *Fra a 1.01* and *Fra a 1.02* were the major paralogs, and they were targeted in further experiments. Muñoz et al. (2010) also reported the highest expression of the *Fra a 1.02* gene at the ripening stage. Kaiser et al. (2016) hypothesized that *Fra a 1.01a* was the potential allergen because of its conservation in both diploid and octoploid species. The magnitudes of the *Fra a 1.01* and *Fra a 1.02* expression levels' correlations with the receptacle and achene were consistent in the RNA-seq and real-time PCR results (Figs. 2, 3). The differences in the relative values might be caused by the sequence coverage of the primers designed for *Fra a 1.02* and the underestimation of the number of *Fra a 1.01* genes in genome database.

Localization of *Fra a 1* in strawberry organs

By eating fresh strawberry, consumers can develop OAS through contact with the fruit (receptacle and achene) and skin, as well as the sepal and peduncle. Farmers are exposed to these organs and also other organs, including leaves, flowers (petals), and pollen. Some strawberry farmers in the Netherlands have shown work-related allergic symptoms (Patiwael et al. 2010). Thus, it is essential to study the localization of the *Fra a 1* protein not just to characterize each isoform, but to avoid organs that trigger allergic symptoms. The expression patterns of *Fra a 1.01* and *Fra a 1.02* were different among organs. The *Fra a 1.01* protein was broadly expressed in strawberry, while *Fra a 1.02* was specifically expressed in the leaf, root, and receptacle. This result was expected based on previous knowledge of homologous PR-10 family proteins. The *Fra a 1* protein belongs to the PR-10 family, which has a response mechanism against diseases that is mainly expressed at the infection site (Liu and Ekramoddoullah 2006). The accumulation of PR-10 in an infected root or vascular bundle has been reported in transgenic tobacco and apple (Pinto and Ricardo 1995; Ziadi et al. 2001; Liu and Ekramoddoullah 2003). During

fruit pathogenesis, the expression levels of *Mal d 1* isoforms in apple fruit were induced by fire blight (*Erwinia amylovora*) (Mayer et al. 2011). During the infection process in strawberry, the major pathogenic bacteria often target leaves, roots, and fruit (Amil-Ruiz et al. 2011). *Phytophthora cactorum*, strawberry pestilence bacteria, raises the expression of allergen-like genes in the roots of *F. vesca* (Toljamo et al. 2016). The accumulation of the *Fra a 1.01* protein occurred mainly in the leaf, root, and receptacle, and the accumulation of *Fra a 1.02* was specific to these organs (Fig. 5), which may be naturally more sensitive to the defensive expression of the PR-10 family than reproductive organs like flowers.

Based on the phylogenetic tree, *Fra a 1.01* and *Fra a 1.02* were closely related, but each had different expression patterns in strawberry organs, indicating the functional variety among isoforms. The binding capacities of specific flavonoids may influence the functions of *Fra a 1.01* and *Fra a 1.02* (Casañal et al. 2013). *Fra a 1.01e* specifically binds to quercetin-3-*O*-glucuronide, while *Fra a 1.02* binds myricetin. An HPLC–MS analysis revealed that strawberry is rich in quercetin-3-*O*-glucuronide, while myricetin was not detected (Mikulic-petkovsek et al. 2012). *Fra a 1.02* may bind myricetin in leaves, which are sites of high *Fra a 1* protein accumulation (Fig. 5). Together with information on pathogenesis, the functional characterization of *Fra a 1* and its correlation with pigment may contribute to breeding hypoallergenic strawberry fruit.

Surprisingly, the correlations between the transcriptional and translational expression levels of both *Fra a 1.01* and *Fra a 1.02* were limited. The expression levels of the *Fra a 1.01* gene and its encoded protein showed opposite trends in the receptacle and achene (Figs. 3, 5). *Fra a 1.02* expression was found in floral organs, but the encoded protein's expression was low (Figs. 3, 5). These results demonstrate the importance of investigating protein expression when evaluating the abundance of major allergens in *F. × ananassa*. The lack of correlation between the transcriptional and translational levels may indicate post-transcriptional control (PTC). The degradation of mRNA and/or protein was expected to be the major mechanism. In the mammalian cell, the average half-life of RNA is 9 h and that of protein is 46 h (Schwanhäusser et al. 2011). According to Maier et al. (2009), the correlation between mRNA and protein vary in different organisms (Pearson correlation coefficient, r_p 0.37–0.76, Spearman rank coefficient, r_s 0.45–0.74 or not determined). The regulation of transcripts by non-coding RNA is another mechanism of PTC. However, to date, there are no data that prove the presence of PTC.

We assumed that the *Fra a 1* protein had the ability to be transported through organs. Inui et al. (2013) identified major latex-like proteins, which are a subfamily of Bet v 1, in the xylem sap of the Cucurbitaceae family. The major latex-like proteins have the ability to bind polychlorinated

biphenyls. The study indirectly demonstrated the transferability of Bet v 1 orthologs and ligand materials. In strawberry, intermediates of flavonoid pigments were indicated as the ligand of the Fra a 1 protein (Casañal et al. 2013). The localized organs containing these flavonoids were identified as the receptacle and achene (Fait et al. 2008). In addition, plant hormones showed similar localizations (Symons et al. 2012). In particular, auxin, which regulates fruit growth and ripening, was synthesized in the achene and then transported to the receptacle (Nitsch 1950). Like other ligands, Bet v 1 can bind various substances, such as physiological ligands, like fatty acids and cytokinins (Mogensen et al. 2002). Thus, ligand candidates must be identified, and the transferability of Fra a 1 through the entire plant body, especially the vascular bundle, must be investigated.

Allergic potential of Fra a 1 based on its abundance in strawberry

The competition assay revealed that anti-Fra a 1.01e antibodies were more abundant than those recognizing specific epitope of Fra a 1.02 in the guinea pig antiserum (Fig. S3). On the other hand, this antiserum showed the similar dose-responses against both Fra a 1.01e and Fra a 1.02 with high concentration at 1:400 dilution (Fig. S4). In this condition, it also had similar reactions to all of the recombinant Fra a 1 proteins (Fig. 4). This reactivity was used to compare the levels of Fra a 1 proteins in strawberry fruits and other organs (Fig. 5). The Fra a 1.01 protein was more abundant than Fra a 1.02 and other isoforms in strawberry fruit and other organs. To our knowledge, this is the first report comparing Fra a 1 isoforms by their expression level in strawberry. Most individuals' IgE mainly reacted with the recombinant Fra a 1.01 protein (Fig. 7). Although the binding levels between IgE and Fra a 1 proteins from strawberry organs were weak, this was probably due to individual differences and detection limits. We assumed that the most important antigen was that of Fra a 1.01 based on the results from transcriptional and translational analyses and the IgE-binding assay. This hypothesis was supported by two previous reports (Musidlowska-Persson et al. 2007; Kaiser et al. 2016), but contradicted by two other reports that assert Fra a 1.02 is the most important antigen (Muñoz et al. 2010; Franz-Oberdorf et al. 2016). Franz-Oberdorf et al. (2016) concluded by both basophil activation tests and gene expression. The cause of divergence in the major allergen may be a difference in methods and/or genotypes. Thus, it remains unknown whether the major allergen in *F. × ananassa* is either Fra a 1.01 or Fra a 1.02, or both. Among *Fra a 1.01* genes, *Fra a 1.01e* gave the highest contribution to both transcriptional and translational expression as shown by the results from database search, RNA-seq, and MS analysis.

We determined that receptacles, achenes, and anthers were the candidate allergenic organs for the occupational-based strawberry allergy. Receptacles and achenes clearly expressed the Fra a 1 protein, while Fra a 1 expression, as well as the binding capacity to human IgE, were hardly detected in anthers, in spite of reacting with birch pollen (Fig. 8). Our results did not indicate that the Fra a 1 protein induced a strawberry pollen-based allergy. In addition to Fra a 1, nonspecific lipid-transfer protein and profilin were identified as other allergic proteins involved in strawberry allergies (Zuidmeer et al. 2006; Hyun and Kim 2011). The sera from patients having work-related allergic symptoms reacted with several proteins (Patiwael et al. 2010), and they may contain some antigens to IgE binding.

Franz-Oberdorf et al. (2016) noted some key mutational points among Fra a 1.04–1.06 based on lower basophil activation levels. Each Fra a 1 protein had amino acid substitutions at various points, thus universal transmutations could not be detected. Furthermore, Gepp et al. (2014) concluded that epitopes are spread across the entire Bet v 1 surface and the IgE recognition profile of Bet v 1 is highly patient specific. Thus, there are limitations when using IgE to evaluate overall allergic reactions. A universal anti-Fra a 1 antibody used in this study may be a practical probe to determine the allergenic potentials of plants.

Conclusion

Fra a 1 protein causes strawberry OAS, and there are many isoforms in *F. × ananassa*. To select the most important targets, we organized Fra a 1 and investigated their expression levels in strawberry organs. RNA-seq indicated that the transcripts of *Fra a 1.01* and *Fra a 1.02* were mainly expressed in fruit. Based on protein translation, Fra a 1.01 was extensively localized in vegetative organs, flowers, fruit, and especially the receptacle. Fra a 1.02 was specifically localized in the leaves, roots, and receptacles. The Fra a 1.01 protein accumulated in the organs to a greater extent than other isoforms. The mRNA expression did not correlate with the protein expression. The reactivity to human IgE against birch was most sensitive to recombinant Fra a 1.01.

Based on the localization, accumulation level and reactivity to human IgE, we concluded that the most important antigens were that of Fra a 1.01 encoded mainly by *Fra a 1.01e*, followed by Fra a 1.02, and then the other Fra a 1 proteins. The information obtained in this study will be useful in breeding hypoallergenic strawberry.

Author contribution statement YU and MIs conceived and designed research. MIs, YU, TN, and YNi conducted experiments. MIs and YU analyzed data. TN, YNi, HT and

MId contributed to data analysis. MIs and YU wrote and revised the manuscript. All authors read and approved the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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A1 (07080)

Protein sequence coverage: 60%

Matched peptides shown in **bold red**.

```
1  MGVTYENEF  TSDIPAPKLF  KAFVLDADNL  I PKI APQAMK  CAB LEEDGG
51  PGII KKI TFG  EGSHYGVMKH  KIHSDIKVNH  TYSYSLIEGD  ALSDNIEKI D
101 YETKLVSAPH GGII I KTTSK  YHTKGDVI K  EBHWAGKEK  ASHLFLU EG
151 YLUKHPSEYN
```

(Fra a 1.01e)

Protein sequence coverage: 56%

Matched peptides shown in **bold red**.

```
1  MGVTYENEF  TSDIPAPKLF  KAFVLDADNL  I PKI APQAMK  CAB LEEDGG
51  PGII KKI TFG  EGSHYGVMKH  KIHSDIKVNH  TYSYSLIEGD  ALSDNIEKI D
101 YETKLVSAPH GGII I KTTSK  YHTKGDVI K  EBHWAGKEK  AAHLFKU EG
151 YLUKHPSEYN
```

R1 (Fra a 1.01e)

Protein sequence coverage: 66%

Matched peptides shown in **bold red**.

```
1  MGVTYENEF  TSDI PAPKLF  KAFVLDADNL  I PKI APQAMK  CAB LEEDGG
51  PGII KKI TFG  EGSHYGVMKH  KIHSDIKVNH  TYSYSLIEGD  ALSDNIEKI D
101 YETKLVSAPH GGII I KTTSK  YHTKGDVI K  EBHWAGKEK  AAHLFKU EG
151 YLUKHPSEYN
```

(07080)

Protein sequence coverage: 66%

Matched peptides shown in **bold red**.

```
1  MGVTYENEF  TSDI PAPKLF  KAFVLDADNL  I PKI APQAMK  CAB LEEDGG
51  PGII KKI TFG  EGSHYGVMKH  KIHSDIKVNH  TYSYSLIEGD  ALSDNIEKI D
101 YETKLVSAPH GGII I KTTSK  YHTKGDVI K  EBHWAGKEK  ASHLFLU EG
151 YLUKHPSEYN
```

R2 (07065)

Protein sequence coverage: 61%

Matched peptides shown in **bold red**.

```
1  MGVTYETEF  TSVIPPPRLF  KAFI LDAADNL  I PKI APQAMK  CAB I EDDGG
51  VGI KKI TFG  EGSGFGSVTH  KIDGIDKNF  VYSYSLIEGD  ALSDKIEKI S
101 YETKLVSDD GGI I KSTSN  YHTKGDVI K  EBHWAGKEK  ASHLFLV EG
151 YLLANPEYC
```

(Fra a 1.02)

Protein sequence coverage: 61%

Matched peptides shown in **bold red**.

```
1  MGVTYETEF  TSVIPPPRLF  KAFI LDAADNL  I PKI APQAMK  CAB I EDDGG
51  VGI KKI TFG  EGSGFGSVTH  KIDGIDKNF  VYSYSLIEGD  ALSDKIEKI S
101 YETKLVSDD GGI I KSTSN  YHTKGDVI K  EBHWAGKEK  ASHLFLV EG
151 YLLANPEYC
```

(04962)

Protein sequence coverage: 42%

Matched peptides shown in **bold red**.

```
1  MGVTYETEF  ISVIPPARLF  KAFI LDAADNL  I PKI APQAMK  GAETIEGDGG
51  VGTIKKITFG  EGSQYSSVTH  RIDGIDKNF  VYNYSIEGD  AISDKIEKI S
101 YETKLVSDD GGI I KSTSN  YHTKGDVI K  EBHWAGKEK  ASHLFLV EG
151 YLLANPEYC
```

(07084)

Protein sequence coverage: 22%

Matched peptides shown in **bold red**.

```
1  MGLFFDQFLS  SHINMGVFT  YETEFTSVIP  PPRLYKAVL  DADNLIPKI A
51  QAMKSAEIV  QDGGGVGTIK  KIHLEGSEY  SYVKHQIDGL  DKDNYVYVYS
101 IIEGDAIGDK  VEKI SYBI KL  VAFSGGSI I  KSTSHYHCKG  EVEIKEEHVK
151 AGKERAAGLF  KI I ENHLAN  FEAYN
```

(Fra a 1.03c)

Protein sequence coverage: 15%

Matched peptides shown in **bold red**.

```
1  MGVTYETEF  TSVIPPPKLF  KAFVLDADNL  IPKI APQAMK  SAEICEGDGG
51  VGTIKKIHLG  EGSEYSYVKH  KIDGLDKDNY  VYNYSIEGD  AIGDKVEKI S
101 YE KLI ASEG  GSI I KSTSHY  HCKGEVEIKE  EHVKAGKERA  AGLFKIVESY
151 LLAHPPEEYN
```

Fig. S1 Positions covered by the Fra a 1 peptide sequences in strawberry (*Fragaria × ananassa*) as determined by an Orbitrap mass spectrometer.

Three proteins were selected based on the results of immunoblotting with anti-Fra a 1e antibody (Figure 5 and Table 1). The positive bands were excised from SDS-PAGE gels, and peptides were digested with trypsin. The digested peptides were introduced into a LTQ Orbitrap Discovery mass spectrometer and identified by peptide mass fingerprinting. Red bold sequences indicate positions that match those of the Fra a 1 proteins. The reference peptide sequences are shown in Table S1.

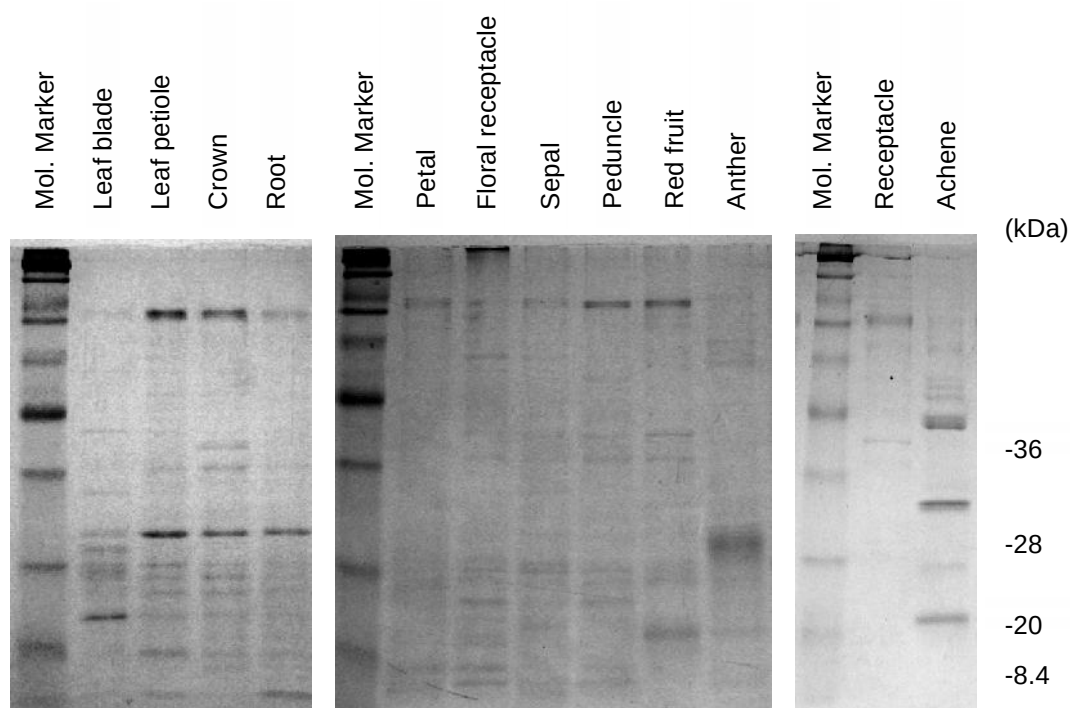


Fig. S2 Each profile of protein in various organ of strawberry (*Fragaria × ananassa*).

Each protein was extracted from leaf blades, petioles, crowns and roots of new stock; petals, floral receptacles, sepals, peduncles and anthers of 5–10 flowers in full bloom from randomly selected individual; and receptacles and achenes of ripe fruit in strawberry cv. ‘Akihime’. 2 µg protein was separated by SDS-PAGE with 15% acrylamide gels, and stained with Coomassie brilliant blue solution (Nacalai Tesque).

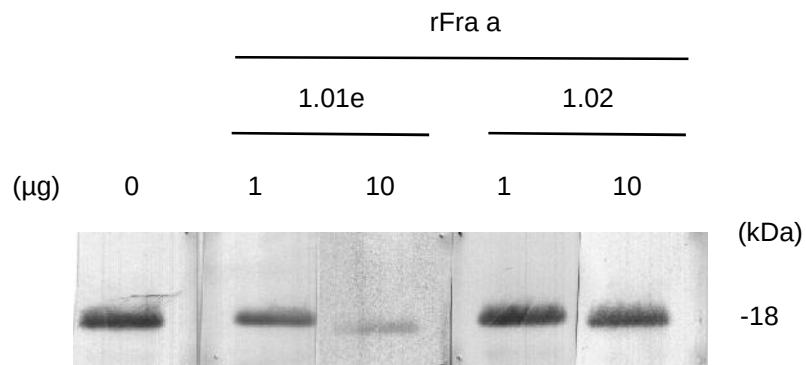


Fig. S3 Competition assay of anti-Fra a 1.01e anti-serum. Using strawberry cultivar ‘Akihime’, proteins were extracted from ripe fruit and analyzed by SDS–PAGE with 15% acrylamide gels. Accumulations of Fra a 1 protein in strawberry fruit (*Fragaria × ananassa*) were detected by immunoblotting. Guinea pig antiserum solution at 1:25,000 dilution, including a polyclonal antibody raised against 6× His-tagged recombinant Fra a 1.01e, was incubated with 1 or 10 μg of 6× His-tagged recombinant Fra a 1.01e or 1.02 proteins before blocking and antibody incubation of blotted PVDF membrane.

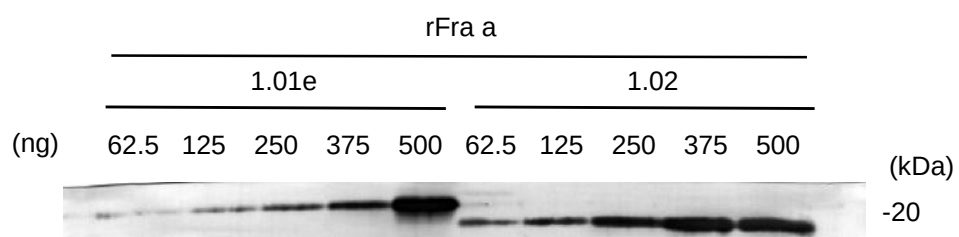


Fig. S4 Dose-response of antibodies to recombinant Fra a 1.01e and 1.02 proteins. The expression and detection of proteins were carried out as described in Figure 4 (guinea pig antiserum including a polyclonal antibody raised against 6× His-tagged recombinant Fra a 1.01e; 1:400 dilution).