



Phosphorylation-dependent ribonuclease activity of Fra a 1 proteins

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ABSTRACT

Abiotic and biotic stress situations cause the upregulation of the transcription of a number of plant defence genes. They code for so-called pathogenesis-related (PR) proteins such as PR proteins of class-10 (PR-10), whose biological functions are still unclear. PR10 proteins are members of the Bet v 1 (major birch pollen allergen) superfamily including related proteins from the cultivated strawberry *Fragaria* × *ananassa* (Fra a 1 proteins). Here, we analyzed the expression of 21 *Fra a 1* genes in different tissues of the strawberry plant by quantitative real-time PCR. Thirteen members were mainly expressed in roots, three in stems, two in red fruits and leaves, and one in flowers. Five genes (*Fra a 1.04–1.08*) were selected based on their expression profiles, heterologously expressed in *Escherichia coli*, and their recombinant proteins functionally characterized. Ribonuclease activity, demonstrated by in-solution and in-gel RNA degradation assays, indicated complete hydrolysis of RNA only by *Fra a 1.06*. Moreover, phosphorylation assays showed that except for *Fra a 1.06*, the remaining four recombinant proteins were phosphorylated. Consequently, we investigated whether the phosphorylation status of the proteins affects their ribonuclease activity. Using an in-solution as well as an in-gel RNase activity assay, results demonstrated that the four recombinant proteins, dephosphorylated with phosphatases, exhibited ribonucleolytic activity against total RNA. Thus, the PR10 related proteins characterized in this study harbour a phosphorylation-dependent RNase activity. The results shed new light on the assumed function of PR10 proteins in plant defence.

1. Introduction

Strawberry is a small fruit crop of great importance throughout the world and appreciated for its unique flavor and nutritious quality. This fruit belongs to the genus *Fragaria* in the Rosaceae family and the Rosoideae subfamily. *Fragaria* consists of 23 species including diploids, tetraploids, hexaploids, and octoploids. Those four basic fertility groups are associated with their ploidy level or chromosome number (Folta and Davis, 2006). The diploid woodland strawberry *Fragaria vesca* or fraise de bois ($2n = 2x = 14$) is the most common native species and is considered as a reference genomic system for the Rosaceae family. *F. vesca* has one of the smallest genomes among cultivated plants, which was estimated at ~240 Mb (Shulaev et al., 2008, 2011; Edger et al., 2017). The woodland strawberry was widely grown until the late 1800s when it was replaced by *F. × ananassa*. The cultivated strawberry, *F. × ananassa*, is an accidental hybrid between *F. chiloensis* and *F. virginiana* that was first found in France in the 18th century by the French Botanist Antoine Nicholas Duchesne. The name *F. × ananassa* was accorded due to the perfume of the fruit that smelled like pineapple (*Ananas*) (Hancock et al., 2008). The genome composition of the octoploid ($2n = 8x = 56$) *F. × ananassa* is among the most complex of the crop species.

Cultivated *F. × ananassa* is grown all over the world but natural populations of *F. × ananassa* are restricted to coastal California, Oregon and Washington whereas *F. vesca* inhabits North and South America, Europe, Asia and Hawaii (Hancock and Luby, 1993). Complete genome sequencing of the woodland strawberry was carried out in 2010 and resequencing in 2018 placing the Rosaceae plant family at the cutting edge of the genomics revolution (Peace and Norelli, 2009; Edger et al., 2017).

Pathogenesis-related (PR) proteins, one of the most commonly expressed proteins during plant defence mechanism, are induced upon pathogen attack, wounding and environmental stresses such as salinity, cold and ultraviolet radiation. PR-proteins have been classified into 17 (PR1–17) families based on sequence similarity and biochemical functions (Radauer et al., 2008; van Loon et al., 2006). The *Fra a 1* allergen in strawberry *F. × ananassa* belong to the group of PR-10 (17 kDa) and are homologous to the major birch pollen Bet v 1 (*Betula verrucosa*) (Breiteneder et al., 1989). Several isoforms of the *Fra a* protein have been described in strawberry, including *Fra a 1E* and *Fra a 3* (Muñoz et al., 2010; Musidlowska-Persson et al., 2007; Casañal et al., 2013a). An NMR structural model of *Fra a 1E* was obtained (Seutter von Loetzen et al., 2014) and crystallization and preliminary X-ray analysis of the

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Fra a 1E and Fra a 3 isoforms were successfully performed (Casañal et al., 2013a). Numerous presumed functions have been assigned to the PR-10 proteins. Besides a proposed ligand binding activity (Mogensen et al., 2002; Neudecker et al., 2001), PR-10 proteins were demonstrated to be involved in enzymatic processes such as ribonuclease activity and are assumed to play a role in secondary metabolite biosynthesis. A ribonuclease was isolated from a callus cell culture of *Panax ginseng* and later shown to be a PR10 protein (Moiseyev et al., 1994). RNase activity was also detected for Bet v 1 (Bufe et al., 1996; Swoboda et al., 1996), white lupin roots LaPR-10 (Bantignies et al., 2000), yellow lupine LiPR-10.1B (Biesiadka et al., 2002), birch pollen BpPR-10c (Koistinen et al., 2002), cotton GaPR-10 (Zhou et al., 2002), seeds of SPE16 (Wu et al., 2002, 2003), hot pepper CaPR-10 (Park et al., 2004), yellow-fruit nightshade SsPR-10 (Liu et al., 2006), peanut AhPR-10 (Chadha and Das, 2006), pea PsPR-10.1, PsPR-10.4 (Srivastava et al., 2006, 2007; Krishnaswamy et al., 2011), *Astragalus mongholicus* AmPR-10 (Yan et al., 2008), peach Pru p 1.01, Pru p 1.06D (Zubini et al., 2009), cacao TcPR-10 (Pungartnik et al., 2009), maize ZmPR-10, ZmPR-10.1 (Chen et al., 2006; Xie et al., 2010), saffron CsPR-10 (Gomez-Gomez et al., 2011), ginseng PgPR-10 s (Lee et al., 2011) and biofuel crop JcPR-10a (Agarwal et al., 2013). The glycine-rich motif was believed to be the most probable nucleotide-binding site of PR-10 and therefore was suggested to bind the phosphate group of RNA (Bantignies et al., 2000). While some PR-10 proteins show RNase activity, others lack this hydrolase activity. In this context, it was proposed that the phosphorylation of the CaPR-10 might increase the RNase activity (Park et al., 2004) and others suggested that the antifungal activity of TcPR-10 might be correlated to its RNase activity (Chadha and Das, 2006; Pungartnik et al., 2009).

Post-translational modifications of the isoforms of Bet v 1 have been deeply studied. Bet v 1 is post-translationally modified by glutathione (Koistinen et al., 2002). The S-glutathiolated cysteine residue is not conserved among Bet v 1 homologous proteins suggesting a unique role for S-glutathiolation of PR-10c compared to the other members of the Bet v 1 family. However, the Cys₈₂ was found in hazelnut (*Corylus avellana*) allergen sharing 85% identity with PR-10c and in *Castanea sativa* plants (Lüttkopf et al., 2002). Some studies established that PR-10s are phosphorylated. It was postulated that phosphorylation of CaPR-10 is a resistance response of plants to biotic stress stimulated by avirulent pathogens and that the ribonuclease function was increased consequently by the phosphorylated state (Park et al., 2004). Some PR-10 proteins from *Arachis hypogaea* were shown to be phosphorylated but their role in RNase activity was not shown (Jain et al., 2006). In contrast, phosphorylation did not influence the RNase activity of TcPR-10 or its substrate specificity and both phosphorylated and non-phosphorylated cocoa PR-10 degraded plant RNA (Pungartnik et al., 2009).

In the present study, we investigated the expression profiles of 21 PR-10 related genes in different tissues of *F. × ananassa* and assessed the ribonuclease activity of five recombinant, putative PR-10 proteins. Because the proteins differed in their phosphorylation status and showed divergent RNase activity, we studied their phosphorylation dependent enzymatic activity by different experimental approaches.

2. Materials and methods

2.1. Plant material

Strawberry *F. × ananassa* cv. Elsanta plants were provided from “Kraege International” company (Telgte, Germany) and were cultivated in a greenhouse (Freising, Germany). Growing conditions were maintained at 25 °C (16 h/8 h, light/dark period).

2.2. First-strand cDNA synthesis

Total RNA of *F. × ananassa* cv. Elsanta was isolated from leaves, young leaves, stems, roots, flowers, as well as from green, white, and

red fruits, as described by Liao et al. (2004) using the CTAB method. In order to remove any DNA contamination, extracted RNA was treated with DNase. One µg of DNase-treated RNA was reverse transcribed to cDNA using 1 µl of random primers (10 µM), 1 µl of 10 mM dNTP and 1 µl of M-MLV RT in 20 µl total volume according to the manufacturer's instruction.

2.3. Quantitative real time PCR (qPCR)

A StepOnePlus™ Real-time PCR system from Applied Biosystems, Californien, USA was used and qPCR analysis was performed according to the manufacturer's instruction. The amplification was carried out as follows in 20 µL total volume: 10 µL Fast SYBR Green Master Mix, 10 µM forward primer (Supplementary material Table S1), 10 µM reverse primer (Supplementary material Table S1) and 2 µL of the template (cDNA). The PCR amplification program was optimized for 40 cycles using the following standard thermal program: 95 °C for 10 min, then 95 °C for 15 s and 60 °C for 1 min (40 cycles), followed by 95 °C for 15 s, 60 °C for 1 min and 95 °C for 15 s. The amplification was carried out in 96-well reaction plate (Applied Biosystems) and all samples were amplified in at least three technical replicates. A cDNA dilution of 1/20 for the target gene and 1/8,000 for the housekeeping gene used for the gene quantification studies. Evaluation of the data with StepOne Software was performed. The Ct value (threshold cycle value) of each *Fra a 1* gene was normalized using the Ct value of the strawberry *FaRIB413* gene, corresponding to an internal RNA interspacer (16S-23S). The fold change of gene expression $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001; Schmittgen and Zakrajsek, 2000) was determined using the following expressions:

$$\Delta Ct = Ct(\text{target gene}) - Ct(\text{internal control gene})$$

$$\Delta\Delta Ct = \Delta Ct(\text{target gene}) - \Delta Ct(\text{reference gene})$$

$$\text{Relative gene expression} = \text{Fold change} = 2^{-\Delta\Delta Ct}$$

2.4. Cloning of *Fra a 1* genes

To amplify the cDNA, a polymerase chain reaction (PCR) was carried out using forward and reverse primers (Supplementary material Table S2). Phusion polymerase was used and PCR reaction was performed in a total volume of 20 µl. The PCR program used was 98 °C for 2 min then 98 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min and finally 72 °C for 10 min. The amplification product was A-tailed and cloned into pGEM-T Easy vector (Promega). The ligation product was transformed into *Escherichia coli* NEB10 beta (New England Biolabs). Bacterial cells were plated out on LB plates, containing the appropriate antibiotics, and incubated for 18 h at 37 °C. The *Fra a 1* genes were digested and ligated into the pre-digested pQE70 vector including a C-terminal Histag (Qiagen, Hilden, Germany). After sequencing (Eurofins), pQE70-*Fra a 1.04* – *1.08* constructs were transformed in *E. coli* BL21 (DE3)pLysS (Novagen, Darmstadt, Germany). A heat shock method was used for the transformation: 1 min at 42 °C in water bath followed by 2 min incubation on ice.

2.5. Heterologous *Fra a 1* protein expression

E. coli BL21 (DE3) pLysS cells harbouring *Fra a 1*-pQE 70 constructs were inoculated into 1 L of LB medium containing antibiotics. Cells were grown under shaking at 150 rpm overnight to an OD₆₀₀ of 0.6–0.8, 1 mM IPTG was added, and proteins produced for 16–20 h. Cells were harvested, disrupted by sonication and recombinant proteins affinity purified.

2.6. Electrophoresis and immunoblotting

The correct sizes of the recombinant Fra a 1 proteins were verified by SDS-PAGE on 12% polyacrylamide gels after coomassie brilliant blue staining. Blotting transfer of Fra a 1 proteins was performed on a polyvinylidene fluorid membrane using a semidry blotter. The incubation with the primary antibody was performed at 4 °C overnight; afterwards, the membrane was rinsed and incubated with the secondary antibody for 1 h at RT. For detection, the membrane was incubated in 20 mL detection buffer, which contained 50 µL of nitro blue tetrazolium and 25 µL of 5-bromo-4-chloro-3-indolyl phosphate in the dark on the shaker, until bands appeared. Finally, the membrane was rinsed with water and scanned.

2.7. RNase activity

2.7.1. In-solution RNase activity

In-solution RNase activity assay was performed by incubating the five recombinant Fra a 1.04–1.08 proteins in the presence of total RNA (1 µg; isolated from strawberry leaves) at different pH values for 4 h at 37 °C. Ten µL of 100 mM citrate buffer at pH 3, MES buffer (2-(N-morpholino) ethanesulfonic acid) at pH 5, phosphate buffer at pH 7.4 and CAPS buffer (N-cyclohexyl-3-aminopropanesulfonic acid) at pH 9 were used. Denatured proteins (boiled at 100 °C for 15 min), empty vector and phosphate buffer alone served as negative controls. RNA was separated on 1% agarose gel stained with ethidium bromide. In-solution RNase activity was also tested after dephosphorylation of Fra a 1.04, Fra a 1.05, Fra a 1.07 and Fra a 1.08 with alkaline phosphatase calf intestinal (CIP) and lambda phosphatase for 1 h at 37 °C and 30 °C, respectively. One µg of the four phosphoproteins was also incubated in the presence of total RNA of strawberry fruit at 37 °C for 4 h (pH 7.4). Fra a 1.06 was used as positive control whereas phosphate buffer and empty pQE70 vector were used as negative control. Controls were also dephosphorylated with CIP for 1 h at 37 °C.

2.7.2. In-gel RNase activity

In-gel RNase activity was tested as described by Yen and Green (1991). The resolving gel was cast with 2.4 mg/mL of yeast torula RNA and an equal volume of 2x sample loading buffer was added to 5 µg of the proteins. Gels were run at a constant voltage of 140 V for 2 h. After one dimensional electrophoresis, SDS gels were washed twice with 25% isopropanol in 0.01 M Tris-HCl and then twice with 0.01 M Tris-HCl to remove the isopropanol, each step 10 min at RT. Following the washing steps, gels were incubated in 0.1 M Tris-HCl for 50 min at 51 °C. Afterwards, gels were stained once with 0.2% toluidine blue in 0.01% Tris-HCl for 10 min and destained twice in 0.01% Tris-HCl. Finally, gels were rinsed, equilibrated in 10% glycerol and dried in cellophane. In-gel RNase activity generate a gel with blue background and proteins with RNase activity were located on the basis of bright region observed at the molecular weight corresponding to the protein of interest. The activity was tested in the presence and absence of reducing agents. In-gel RNase activity was also performed with 10 µg of the 4 dephosphorylated Fra a 1.04, Fra a 1.05, Fra a 1.07 and Fra a 1.08 proteins, produced by treatment with alkaline phosphatase calf intestinal (CIP) and lambda phosphatase for 1 h at 37 °C and 30 °C, respectively.

2.8. Phosphorylation assay

2.8.1. Protein gel blot analysis

Equal amounts of the five Fra a 1.04–1.08 proteins (10 µg) were separated by SDS-PAGE and blotted to a polyvinylidene fluorid membrane. For the blocking buffer, blot-qualified bovine serum albumin (BSA) dissolved in washing buffer was used. The membranes were probed with phosphoserine/-threonine/-tyrosine (SPM101) monoclonal antibody from Invitrogen as primary antibody at a 1:1000 dilution. After an overnight incubation at 4 °C, the membrane was washed

and incubated at RT for 1 h with an anti-mouse IgG conjugated to alkaline phosphatase as a secondary antibody at a 1:30,000 dilution. Detection step was carried out overnight under gentle agitation at room temperature. In this assay, pepsin was used as a positive control and lysozyme as a negative control.

2.8.2. Pro-Q Diamond phosphoprotein Gel Stain

The putative Fra a 1 phosphoproteins were stained with Pro-Q Diamond fluorescent gel according to the protocol of the manufacturer (Invitrogen). All steps of staining, destaining and washing were performed using gentle agitation on an orbital mixer (50 rpm). After polyacrylamide electrophoresis, the gel was fixed in 100 mL of a fix solution (50% methanol, 10% acetic acid) and incubated overnight at RT. Then, in order to remove residual methanol and acetic acid, the gel was washed 3 times in 100 mL of ultrapure water for 10 min. After a staining step with Pro-Q Diamond Gel Stain for 90 min in the dark, the gel was destained in 100 mL destain solution (20% acetonitrile, 50 mM sodium acetate, pH 4) for totally 90 min at room RT. Finally, the gel was washed twice for 10 min each in ultrapure water. The proteins were visualized using a UV transilluminator box at an excitation wavelength of 300 nm.

2.8.3. Sypro Ruby Gel Stain

After staining with Pro-Q Diamond phosphoprotein Gel Stain, the gel was subsequently stained overnight in the dark with a total protein stain Sypro Ruby, which is an organometallic ruthenium chelate stain designed for detection applications. Then, the gel was destained with a destain solution (10% methanol, 7% acetic acid) for 30 min and washed twice in ultrapure water. A 300 nm UV box was used for the detection.

2.8.4. Chemiluminescent assay

Protein gel blot analysis was performed by loading equal amounts of the five Fra a 1 proteins onto an SDS-PAGE gel. Proteins were transferred to a PVDF membrane and blocked with 5% BSA in washing buffer for 1 h at RT. The membranes were probed with phosphoserine/-threonine/-tyrosine monoclonal antibody (SPM101) at 1:700 dilution. After an overnight incubation at 4 °C, the membrane was washed and incubated at room temperature for 1 h with a Goat anti-mouse IgG horseradish peroxidase conjugate (HRP) as a secondary antibody at 1:9500 dilution. A substrate working solution was prepared by mixing equal parts of two chemiluminescent substrates: the peroxide solution and the luminol enhancer solution. After an incubation time of 1 min at RT, the membrane was removed from the solution, blotted with absorbent filter paper to absorb excess liquid and placed in a clear plastic wrap. The membrane was then developed in a dark room and the x-ray film was exposed on the top for around 30 min. Pepsin was used as positive control for this assay and lysozyme as negative control.

3. Results and discussion

3.1. Sequences and structural properties of Fra a 1 proteins

The availability of the complete *F. vesca* genome sequence has made it possible to identify 21 genes similar to *Fra a 1* in the *F. vesca* genome sequence by *in silico* analysis (Shulaev et al., 2011; Edger et al., 2017). Likewise, at least eighteen *Mal d 1* genes of the multigene family of plant PR-10 proteins were identified in apple (Gao et al., 2005), eight PR-10 isoforms in yellow lupine (Handschuh et al., 2007), five PR-10 genes in pea (Tewari et al., 2003), and eight *Pru p 1* and *Pru d 1* isoforms in peach and almond, respectively (Chen et al., 2008). Orthologous *F. vesca* genes encoding for *Fra a 1* isoforms show an open reading frame (ORF) from 399 to 639 bp. Genes *FvH4_4g18990.1*, *FvH4_4g19060.1.b* and *FvH4_4g19010.1* corresponding to *Fra a 1.01*, *1.02*, and *1.03* (Muñoz et al., 2010), respectively show a length of 480 to 483 nucleotides and encode polypeptides of 159 to 160 amino acids. The highly similar genes *FvH4_4g19710.1*, *FvH4_4g19060.1.a*,

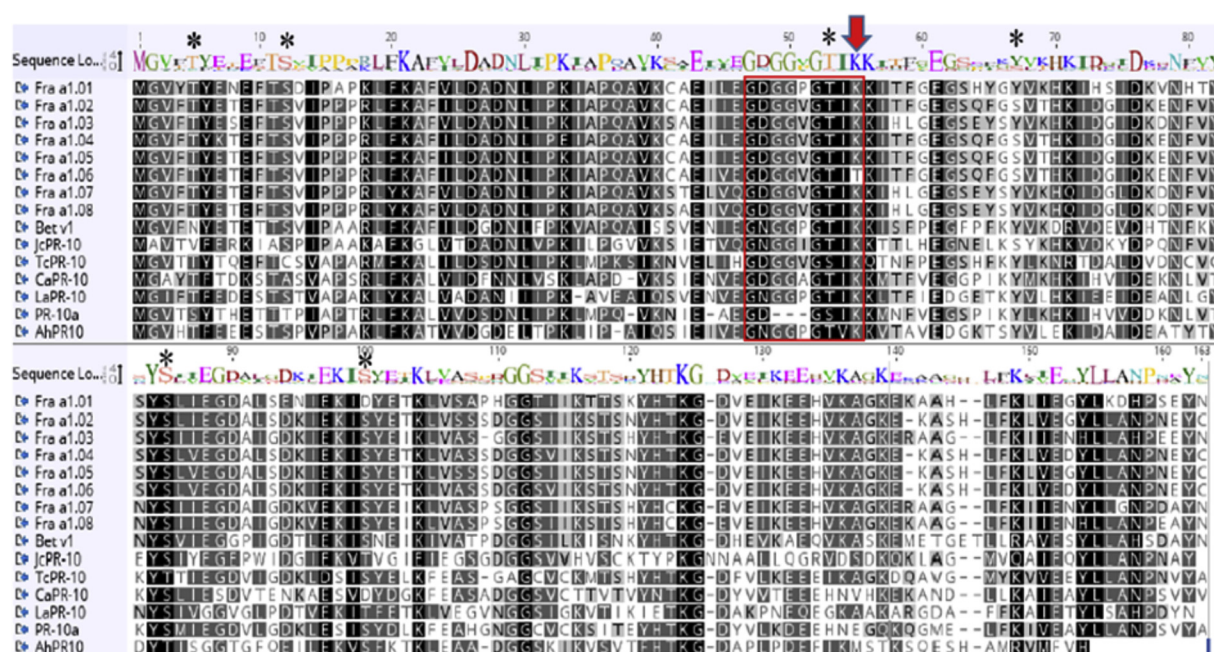


Fig. 1. Protein sequences comparison of Fra 1.01–1.08 with those of Bet v 1, JcPR-10 (biofuel crop), TcPR-10 (cacao), CaPR-10 (hot pepper), LaPR-10 (white lupin), AhPR-10 (peanut) and PR-10a (potato). Amino acids Thr₅, Ser₁₂, Thr₅₃, Tyr₆₇, Ser₈₅ and Ser₁₀₀ are predicted phosphorylations sites for Fra a 1 proteins. Phosphorylation sites are marked with an asterisk. The red arrow shows the amino acid distinguishing Fra 1.06 from the other proteins (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

FvH4.4g19080.1, *FvH4.4g19700.1*, and *FvH4.4g18850.1*, whose orthologues in *F. × ananassa* were assigned to *Fra a 1.04*, *1.05*, *1.06*, *1.07*, and *1.08*, respectively are intronless, have an open reading frame of 483 bp and encode polypeptides of 160 amino acids (Fig. 1). Those five *Fra a 1* genes were later chosen for detailed analysis. SDS-PAGE analyses of the recombinant proteins revealed a high degree of purity and protein bands were consistent with the expected molecular weights of about 18 kDa (Supplementary material Figure S1 and Figure S2). The intracellular PR-10 (IPR-10) proteins usually have an ORF encoding a polypeptide of 151–162 amino acids with molecular masses of 15–18 kDa (Supplementary material Figure S3). Interestingly, the proteins encoded by genes *FvH4.4g18970.1* (23.96 kDa), *FvH4.4g19070.1* (21.73 kDa), *FvH4.4g19040.1* (19.36 kDa), and *FvH4.4g18830.1* (14.37 kDa) exceeded this size. Likewise, (S)-norcoclaurine synthase PR-10 NCSs, which exhibit 28–38% identity and putative structural homology with the Bet v 1 protein family (Samanani et al., 2004), has an ORF of 630 bp encoding a protein with a molecular weight of 23.3 kDa.

An alignment of the amino acid sequences of the 21 translated, orthologous *Fra a 1* genes extracted from the *F. vesca* genome and Bet v 1, identified the most similar and most divergent regions (Supplementary material Figure S3). The glycine-rich loop motif GxGGxGxxK is highly conserved throughout the Bet v 1 superfamily (Saraste et al., 1990) and is highlighted by a red box. This consensus sequence was highly conserved in eighteen of the orthologous *Fra a 1* proteins. For the protein encoded by gene *FvH4.4g19080.1* (orthologue of *Fra a 1.06*) the residue at position 55, which is conventionally a lysine (K₅₅) was replaced by a threonine (T₅₅) (Fig. 1). There was one AA exchange for *FvH4.4g18960.1b* (GxGGxGxxE) and *FvH4.4g18960.1a* proteins (GxGExGxxK) while no P-loop motif was detected in the *FvH4.4g18970.1* amino acid sequence (Supplementary material Figure S3). This motif is found in ATP and GTP-binding proteins but not present in all of them. The paralogous *Fra a 1.01–1.08* proteins showed 53.1% to 59.3% amino acid pairwise identity to Bet v 1 (Supplementary material Table S3). Making a restricted comparison within the 21 isoforms, *Fra a 1.01–1.08* proteins showed a pairwise similarity of 69.4 to 99.4% (Supplemental material Table S4).

Furthermore, the eight sequences of *Fra a 1.01–1.08* featured the Bet v 1 motif, which is characteristic for the pathogenesis related proteins of the Bet v 1 family (Supplementary material Figure S3).

Four amino acids have been reported to be invariable across the PR-10 family (Liu and Ekramoddoullah, 2006), which were also identified for *Fra a 1.04–1.08*: Gly₄₇, Gly₅₂, Asp₇₆ and Leu₁₅₅ (Fig. 1). The amino acids facing the cavity in *Fra a 1E*, *Fra a 2* (identical to *Fra a 1.02*) and *Fra a 3* (identical to *Fra a 1.03*) proteins are generally conserved but variability could be observed at certain positions explaining their different selectivity toward ligands (Casañal et al., 2013b). It was shown that key amino acids Leu₅₉ and Arg₁₃₉, involved in *Fra a 3* (+)-catechin interaction were replaced by Phe₅₉ and Lys₁₃₉ in both *Fra a 1E* and *Fra a 2*. The amino acid Phe₅₉ was also conserved in *Fra a 1.04–1.06* and changed to Leu₅₉ in *Fra a 1.07–1.08*, while the arginine residue was detected for *Fra a 1.07* at position 140 and was replaced by Lys₁₄₀ for *Fra a 1.04–1.06* and *Fra a 1.08* proteins. Further important residues detected by Casañal et al. (2013b) such as His₇₀ and Tyr₈₄ were also identified for *Fra a 1.04–1.08*. PR-10 proteins are generally very similar within species and more distant from protein copies of other plant species suggesting that the homogeneity of the PR-10 multigene family was maintained by concerted evolution (Wen et al., 1997). This phenomenon causes genes to evolve as a single unit with members exchanging genetic information through gene conversion and unequal crossing-over. Such a high interspecific but low intraspecific variability has been observed in *Passiflora*, *Betula*, and *Vitis* genus (Finkler et al., 2005; Lebel et al., 2010; Schenk et al., 2009).

3.2. *Fra a 1* genes expression

In order to evaluate the relative expression levels of the 21 *Fra a 1* isoforms in different tissues of the *F. × ananassa* strawberry plant (root, stem, leaf, young leaf, flower and fruit), quantitative real-time PCR analyses were performed. Specific primers from the untranslated region (5' UTR) of each of the 21 genes were designed (Supplementary material Table S1). Contrastingly, the design of specific primers for *Vitis vinifera* PR-10 gene transcripts was not possible even in the 5' and 3' UTRs as a consequence of the high similarity of *VvPR-10.1-a* and *VvPR-*

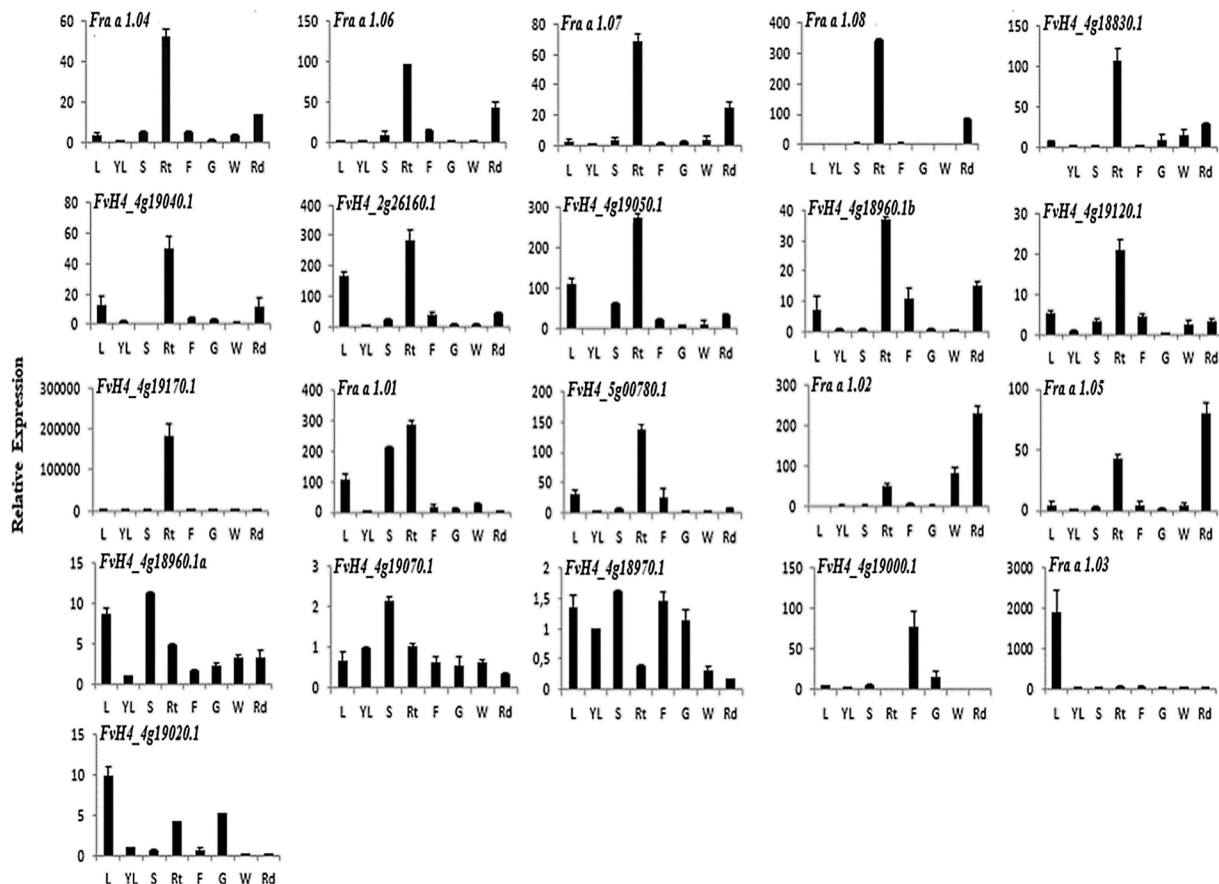


Fig. 2. Relative gene expression of 21 *Fra a 1* genes in different tissues (L) Leaf; (YL) Young leaf; (S) Stem; (Rt) Root and (F) Flower) of *Fragaria × ananassa* cv. Elsanta at various stages of maturity of fruit ((G) Green; (W) White and (Rd) Red). The qPCR data were normalized to the interspacer gene *FaRIB413*. Young leaf was used as reference and values were set to 1. Values are mean of five biological replicates and are shown as relative changes. The error bars indicate the standard deviation of the means.

10.3-a (Lebel et al., 2010). Five biological replicates were analyzed and the results were normalized with an inter-spacer 16–23 S RNA house-keeping gene (*FaRIB413*). As one of the best known and most frequently used reference genes in plants, *FaRIB413* is highly recommended for relative quantification of gene expression in strawberry (Amil-Ruiz et al., 2013). In fact, the *FaRIB413* RNA has been established to be a convenient internal control for gene expression studies in strawberry across several tissues and experimental conditions using qPCR analyses (Benítez-Burraco et al., 2003).

A huge variation in relative transcript levels between the 21 isoforms was observed in all investigated *F. × ananassa* organs (Fig. 2). The expression levels varied from 182,670 fold of gene *FvH4_4g19170.1* in roots to 0.17 fold for gene *FvH4_4g18970.1* in red fruits. Thirteen isoforms were highly expressed in roots. *Fra a 1.04* (52 fold), *Fra a 1.06* (97 fold), *Fra a 1.07* (69 fold), *Fra a 1.08* (347 fold), gene *FvH4_4g18830.1* (106 fold) and gene *FvH4_4g19040.1* (50 fold) were strongly transcribed in roots with an intermediate expression in the late stage of fruit ripening. A comparable pattern was detected for gene *FvH4_2g26160.1* (283 fold) and gene *FvH4_4g19050.1* (275 fold) with a notable expression in leaves. Genes *FvH4_4g18960.1b* (37 fold) and *FvH4_4g19120.1* (20 fold) were also significantly expressed in roots and showed considerable transcript levels in leaves, flowers and red fruits. In contrast, gene *FvH4_4g19170.1* was exclusively transcribed in roots with the highest expression value within the 21 isoforms. *Fra a 1.01* (287 fold) and gene *FvH4_5g00780.1* (136 fold) were predominantly expressed in roots with an intermediate transcript level in leaves for both of them and in stems for *Fra a 1.01*. The high transcript abundance in roots is in line with a putative role in plant defence. Notably, *Fra a 1.02* (231 fold) and *Fra a 1.05* (80 fold), which exhibit 99.4% of protein

sequence similarity (Supplementary material Table S4), were highly transcribed in the late stage of fruit ripening with a lower expression in roots. In contrast, gene *FvH4_4g18960.1a* (11 fold), gene *FvH4_4g19070.1* (2 fold) and gene *FvH4_4g18970.1* (1.6 fold) were expressed in all tissues with relatively higher transcript levels in stems. The high transcript levels of *Fra a 2* in red fruit (Muñoz et al., 2010) is probably connected with the development of an allergic impact on individuals affected by allergy to red strawberries (Franz-Oberdorf et al., 2016). This isoform was identified as an important strawberry allergen of the Bet v 1 superfamily (Franz-Oberdorf et al., 2016; Ishibashi et al., 2017a). Moreover, the transcript levels of *Fra a 1* orthologues in *F. vesca* was investigated by RNA-sequencing (RNA-seq), which was performed on receptacle and achene tissues of three *F. vesca* botanical forms (Yellow Wonder “YW”, Hawaii 4 “HW4” and Reine des Vallées “RdV”) at three different ripening stages (green, white and ripe). *Fra a 1.01* was significantly expressed in all genotypes except ripe receptacle while *Fra a 1.02* was notably expressed at ripening stage in achenes and receptacles. Other studies established that *Fra a 1.02* was mostly expressed in receptacles, and *Fra a 1.01* in achenes (Ishibashi et al., 2017a) whereas *Fra a 1.01* was highly expressed in immature fruit and *Fra a 1.02* in young to ripe fruit (Ishibashi et al., 2017b).

Gene *FvH4_4g19000.1* (76 fold) was a unique *Fra a 1* isoform, which was predominately expressed in flowers with a moderate transcription level in green fruits. *Fra a 1.03* (1,875 fold) was exclusively transcribed in leaves whereas gene *FvH4_4g19020.1* (9 fold) was considerably expressed in leaves and also in roots and green fruits. Altogether, the qPCR analysis revealed that the 21 isoforms are distinctly and variably expressed within *F. × ananassa* tissues with a predominance of roots tissues and a significant expression level at the late stage of fruits

ripening (Supplementary material Figure S4). Similarly, the *Mal d 1* genes were expressed in different tissues while some were expressed in the same tissue, namely in ripe fruit skins (*Mal d 1b*, *d – h* and *i*) or in leaves from disease-challenged seedlings (*Mal d 1b*, *d – f*, *i* and *j*) (Beuning et al., 2004). In general, PR-10 proteins are assumed to show increased gene expression in stress situations. Such expression of stress-induced proteins is triggered by pathogen infection (Swoboda et al., 1995), plant hormones (Wang et al., 1999), wounding (Liu et al., 2005), salt stress (Jain et al., 2006) or cold (Yu et al., 2000). Likewise, in the common morning glory (*Ipomoea purpurea*) of Convolvulaceae the expressions of PR-10 genes were susceptible to both developmental and environmental changes (Lu et al., 2009).

3.3. Heterologous expression of *Fra a 1* proteins

Fra a 1.04–1.08 were selected for further analysis due to their high transcript abundance in both roots and red ripe *F. × ananassa* fruit (Fig. 2; Supplementary material Figure S4). The full-length coding sequences of the five isoforms *Fra a 1.04–1.08*, which were identical to the sequences of *F. vesca* were cloned into pQE70 vector, that contained a 6x His-tag at the C-terminal end. Specific primers amplifying a nucleotide sequence of 483 bp length were designed (Supplementary material Table S2). His-*Fra a 1* proteins were successfully expressed in *E. coli* BL21 (DE3) pLysS cells, which are suitable for high level induction and expression of genes regulated by a T7 promoter. SDS-PAGE analysis of the samples purified by immobilized metal affinity chromatography (IMAC) (Supplementary material Figure S1) indicated bands at about 18 kDa (calculated values: 17.5 kDa for *Fra a 1.04*, 17.5 kDa for *Fra a 1.05*, 17.5 kDa for *Fra a 1.06*, 17.6 kDa for *Fra a 1.07* and 17.5 kDa for *Fra a 1.08*). All the cited proteins revealed a high degree of purity in the eluted fractions. A protein gel blot analysis of the five *Fra a 1* proteins was also performed using the monoclonal anti-6x His Alkaline Phosphatase (AP) antibody. The immunodetection confirmed the results obtained with the Coomassie blue staining of the five *Fra a 1* proteins (Supplementary material Figure S2).

3.4. Ribonuclease activity

3.4.1. In-solution RNase activity

Most of the published reports on PR-10 proteins are based on their amino acid sequence similarity with two ginseng ribonuclease proteins (PgPR-10s) suggesting that this family exhibits ribonuclease activity (Moiseyev et al., 1994). Nucleolytic activity has been shown in *in vitro* test for Bet v 1 (Bufer et al., 1996; Swoboda et al., 1996) as well as for PR-10 proteins from white and yellow lupine (Bantignies et al., 2000; Biesiadka et al., 2002). The in-solution RNase activity of *Fra a 1* proteins was assayed by incubating the proteins in the presence of total RNA isolated from strawberry fruit at different pH for four hours. Ribonucleolytic activity was demonstrated on agarose gel by the migration of the degraded products (Fig. 3). Under acetic conditions (pH 3), the RNA appeared to be completely hydrolyzed by *Fra a 1.06* and partially degraded when incubated with the four remaining *Fra a 1* proteins as well as with the buffer. Thus, RNA becomes chemically degraded after prolonged incubation at pH 3. At pH 5, total RNA degradation was detected for *Fra a 1.06* after only a few minutes whereas *Fra a 1.07* and *Fra a 1.08* were unable of full hydrolysis even after 4 h of incubation. No hydrolysis was detected for *Fra a 1.04* and *Fra a 1.05*. Under basic conditions (pH 7.4 and pH 9), total RNA was completely hydrolyzed after a few minutes only for *Fra a 1.06*. For the rest of *Fra a 1* proteins, no hydrolysis was observed (Fig. 3). No RNA degradation was detected when RNA was incubated with the buffer alone, with the lysate of *E. coli* transformed with pQE70 vector or with *Fra a 1.06* thermally inactivated by incubation at 100 °C for 30 min (Fig. 3). Similarly, LIPR-10.1B from yellow lupine manifested RNase activity while its homologous LIPR-10.1A did not (Biesiadka et al., 2002). Moreover, a PR-10-like protein from white lupin roots required six hours of

incubation time for total hydrolysis of RNA (Bantignies et al., 2000), a pea PR 10.1 needed three hours (Srivastava et al., 2006) while Pru p 1 catalyzed the total hydrolysis within thirty minutes to two hours (Zubini et al., 2009).

3.4.2. In-gel RNase activity

The ribonuclease activity of *Fra a 1.04–1.08* was also evaluated in polyacrylamide gel by incubating the five proteins with yeast torula RNA. A clear band of 18 kDa was observed for *Fra a 1.06* whereas no detectable clear region at the corresponding molecular weight was visible for the rest of the four *Fra a 1* proteins (Fig. 3). Boiled *Fra a 1.06* protein was used as a negative control. Thus, only *Fra a 1.06* possessed RNase activity confirming the result of in-solution RNase activity.

The P-loop structure (GxGGxGxxK) has been considered as the possible RNA phosphate-binding site correlated with ribonucleolytic activity (Bantignies et al., 2000). The conserved RNase binding site P-loop structure in Bet v 1 protein was found in the *Fra a 1* (Supplementary material Figure S3) and PR-10 proteins (Fig. 1). The P-loop motif of the *Fra a 1.04–1.08* proteins was identical from position 47 to 54 (G47D48G49G50V51G52T53I54K55), except for *Fra a 1.06*, which showed a Thr (T) at position 55 (Fig. 1).

In PR-10 proteins with RNase activity, tyrosine (Tyr/Y) and glutamic acid (Glu/E) residues are generally conserved (Fig. 1). In Bet v 1, Glu₉₆, Glu₁₄₈, and Tyr₁₅₀ are possibly implicated in its ribonuclease activity (Moiseyev et al., 1997). The same three amino acids Glu₉₇, Glu₁₅₀ and Tyr₁₅₂ together with the P-loop were implicated in the RNase activity of the JcPR-10a (*Jatropha curcas*; Agarwal et al., 2013) and TcPR-10 (*Theobroma cacao*) protein (Pungartnik et al., 2009). The P-loop domain might be related, but not essential to RNase activity in GbPR-10 from cotton (*Gossypium arboreum*) as in particular, Gly₅₁, Lys₅₅, and Glu₉₆ affected this activity (Zhou et al., 2002). Substitution of Tyr₁₅₀ and Glu₁₄₈ in GaPR-10 remarkably decreased the RNase activity and were replaced in AhPR-10 with Phe₁₄₈ and His₁₅₀, respectively (Chadha and Das, 2006). Also in AhPR-10, Lys₅₄ appeared to play a critical role in the RNase and antifungal activity of the peanut protein. In *Fra a 1.06* protein, the P-loop domain (aa 47–55) along with Glu₉₇, Glu₁₄₉ and Tyr₁₅₁ (Fig. 1) residues could be suggested to play a role in its RNase activity. Interestingly, *Fra a 1.06* differed in only one amino acid from the other four putative PR-10. In *Fra a 1.06* Lys₅₅ is replaced by Thr, which eventually confers RNase activity to the protein. The exact mechanisms underlying the reaction remains to be elucidated.

Since PR-10 proteins are induced by biotic as well as abiotic stresses, their ribonuclease activity suggested a possible role in defence against pathogenic infections in order to protect plants during programmed cell death (PCD) at and around the plant infection sites or to act directly on the pathogen (Liu and Ekramoddoullah, 2006). Besides the ability to inhibit growth of the oomycete pathogen *Phytophthora capsici*, CaPR-10 protein also exhibited ribonuclease activity against tobacco mosaic virus RNA (Park et al., 2004). It was demonstrated that the ribonuclease activity of SsPR-10 (yellow-fruit nightshade) and the peanut AhPR-10 played a crucial role during viral attack (Chadha and Das, 2006; Liu et al., 2006). In addition, the correlation of *in vitro* RNase activity with antimicrobial activity of PR-10 proteins was reported in many reviews. A high specific RNase activity of the maize ZmPR-10.1 protein was established to be consistent with strong antimicrobial activity (Xie et al., 2010). Interestingly, only ribonuclease activity was revealed for Bet v 1 (Bufer et al., 1996; Swoboda et al., 1996), LaPR-10 (Bantignies et al., 2000), and GaPR-10 (Zhou et al., 2002). RNase activity of PR-10 proteins was also linked to antifungal activity. Ribonucleolytic activity of the TcPR-10 protein was correlated with its antifungal activity against the cacao pathogen *M. perniciosa* (Pungartnik et al., 2009). Similarly, AhPR-10 appeared also to exhibit an antifungal activity through its RNase function (Chadha and Das, 2006). Likewise, a *Panax notoginseng* RNase showed antifungal activity (Lam and Ng, 2001). In tobacco, the involvement of plant RNase activity in defence against pathogenic fungus invasion was observed (Galiana et al., 1997).

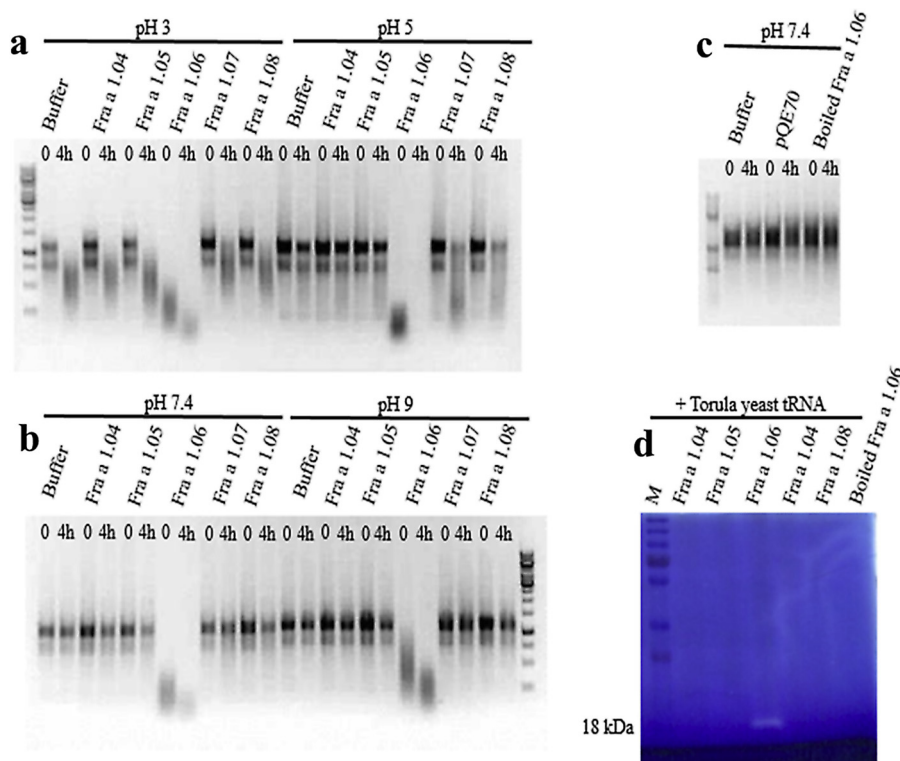


Fig. 3. RNase activity assays of Fra a 1 proteins. In-solution ribonuclease activity of the recombinant Fra a 1.04–1.08 proteins (1 µg) tested against total RNA from strawberry fruits (1 µg) at different pH (3 and 5) (a), (7.4, and 9) (b) and different incubation times (0 and 4 h) at 37 °C. RNA hydrolysis products were separated using agarose gel. Negative controls were also evaluated (c). In-gel RNase activity of Fra a 1.04–1.08 proteins (5 µg) (d). Polyacrylamide gel containing yeast torula RNA was used for electrophoresis. After staining with toluidine blue, a unique clear band was detected demonstrating that only Fra a 1.06 possesses the RNase activity. M: molecular weight marker (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

However, for ZmPR-10, the relationship between its RNase activity and its strong resistance to *Aspergillus flavus* is not fully understood and still needs more clarification (Chen et al., 2006).

Other factors can influence the RNase activity. In the presence of zeatin, the RNA hydrolase activity of the peach major allergen Pru p 1.01 was shown to be considerably affected while no effect was detected for its homologous Pru p 1.06D. In addition, post-translational modifications can affect this catalytic reaction. Phosphorylation of CaPR-10 increased its ribonuclease function because the 19 kDa phosphorylated form of CaPR-10 has 12.4 fold higher RNase activity compared to the 18 kDa unphosphorylated form in degradation assays using yeast total RNA as substrate (Park et al., 2004).

3.5. Phosphorylation assay

3.5.1. Protein gel blot analysis of Fra a 1 proteins

It has long been reported that PR-10 proteins are post-translationally modified by phosphorylation (Ziadi et al., 2001) and that phosphorylation is required for defence response to microbial pathogen attack and stress (Park et al., 2004). Phosphorylation sites were found in hot pepper CaPR-10 (Park et al., 2004), cacao TcPR-10 (Pungartnik et al., 2009), potato PR-10a (Despres et al., 1995), peanut AhPR-10 (Chadha and Das, 2006; Parthibane et al., 2012), white lupin LaPR-10 (Bantignies et al., 2000) and Jatropha JcPR-10a (Agarwal et al., 2013). Therefore, a protein gel-blot analysis (Goodman et al., 2004; Sun et al., 2007) of the five recombinant proteins was performed using a primary antibody raised against phosphoserine/-threonine/-tyrosine and an anti-mouse IgG conjugated to alkaline phosphatase as a secondary antibody (Fig. 4). Except for Fra a 1.06, which possessed ribonuclease activity, a band of about 18 kDa appeared for the recombinant proteins proving their phosphorylation. The bands visible for Fra a 1.06 and 1.07, below 18 kDa are unknown (Supplementary material Figure S5). Treatment of Fra a 1.08 with alkaline phosphatase calf intestinal (CIP) clearly decreased the intensity of the signal indicating a loss of phosphates. Lysozyme and pepsin (tested at two different concentrations) were used as negative and positive controls, respectively and were

corroborating the results (Supplementary material Figure S5).

3.5.2. Pro-Q Diamond and Sypro Ruby Gels Stain

In order to substantiate the protein gel-blot outcome, a Pro-Q Diamond Phosphoprotein Gel Stain (Choi et al., 2012; Goodman et al., 2004; Jain et al., 2006), which can detect phosphoproteins on SDS polyacrylamide gel by a mechanism that combines a chelating fluorophore and a transition metal ion, was performed. Fluorescent detection of phosphoserine, phosphothreonine and phosphotyrosine was possible due to excitation at 555–585 nm. After one-dimensional electrophoresis, the gel was stained with Pro-Q diamond in the dark and then destained. Except for Fra a 1.06, the four remaining Fra a 1 proteins bound the dye and produced clear signals confirming phosphorylation of the proteins Fra a 1.04, Fra a 1.05, Fra a 1.07 and Fra a 1.08 (Fig. 4, Supplementary material Figure S6). After staining with Pro-Q Diamond Phosphoprotein Gel Stain, the gel was subsequently stained with a total protein stain to visualize total proteins (Fig. 4, Supplementary material Figure S6).

3.5.3. Chemiluminescent assay

As a third test, a chemiluminescent detection kit for the visualization of phosphoproteins (Park et al., 2004; Sun et al., 2007), which yields the greatest sensitivity of any available detection method, was also performed. The proteins were first probed with phosphoserine/-threonine/-tyrosine monoclonal antibody, visualized with an anti-Mouse IgG antibody, which is conjugated to the horseradish peroxidase and then incubated with chemiluminescent substrates. Those substrates are two component-systems consisting of a stable peroxide solution and an enhanced luminol solution. After the incubation step, a sheet of film was developed in a dark room. The exposure time was 30 min. The bands at 18 kDa affirmed once again that Fra a 1.04, Fra a 1.05, Fra a 1.07 and Fra a 1.08 are phosphorylated (Fig. 4). Pepsin and lysozyme served as positive and negative control, respectively (Supplementary material Figure S7).

Phosphorylation profile study of the Fra a 1 proteins and Bet v 1 (Basharat, 2016) using the NetPhos 3.1 server (<http://www.cbs.dtu.dk/>

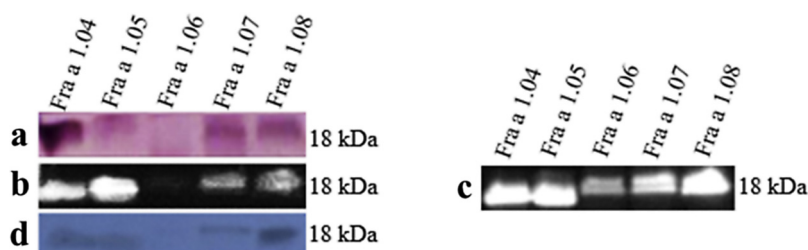


Fig. 4. Phosphorylation assays. Protein gel blot analysis using a phosphoserine/-threonine/-tyrosine antibody for the detection of phosphorylated Fra a 1.04–1.08 proteins (a). Fra a 1.04, 1.05, 1.07 and 1.08 were shown to be phosphorylated due to the bands at 18 kDa. Phosphoprotein detection of Fra a 1.04–1.08 proteins using Pro-Q Diamond staining gel (b). Only Fra a 1.04, Fra a 1.05, Fra a 1.07 and Fra a 1.08 were demonstrated to be phosphorylated. Gel was subsequently stained for total proteins detection with Sypro Ruby stain (c). Chemiluminescent assay of Fra a 1.04–1.08 (10 µg) (d). Horseradish Peroxidase-conjugated antibody and an enhanced luminol-based chemiluminescent substrate were used for the chemiluminescent protein blotting detection. Exposure to X-ray film indicated that Fra a 1.04, Fra a 1.05, Fra a 1.07 and Fra a 1.08 are phosphorylated.

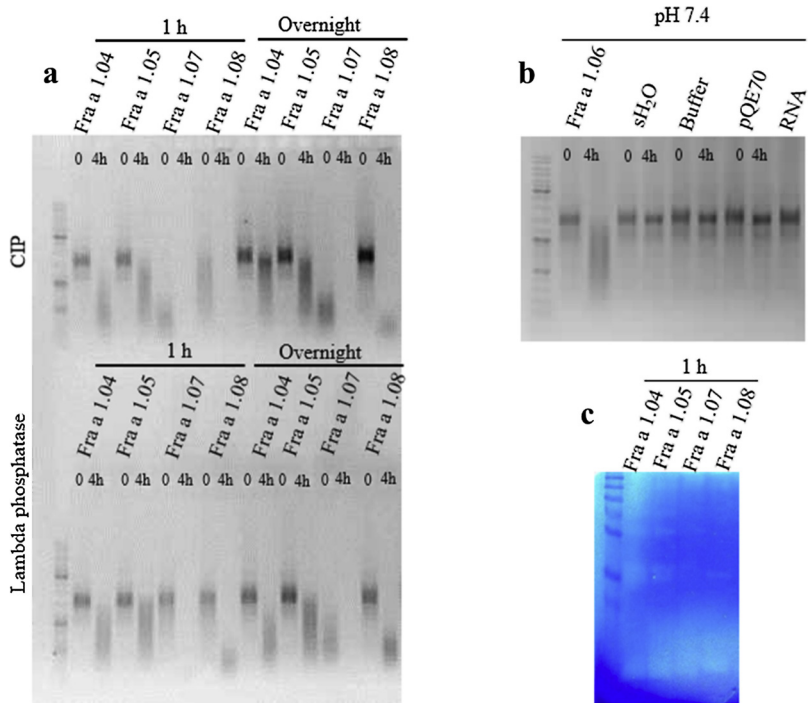


Fig. 5. RNase activity assays of dephosphorylated proteins. In-solution RNase activity of the dephosphorylated Fra a 1.04, Fra a 1.05, Fra a 1.07 and Fra a 1.08 (a). Proteins were dephosphorylated for 1 h and overnight with calf intestinal alkaline phosphatase (CIP) and lambda phosphatase at 37 °C and 30 °C respectively. After dephosphorylation, Fra a 1.04, Fra a 1.05, Fra a 1.07 and Fra a 1.08 were shown to possess RNase activity. Negative (sH₂O, buffer, empty vector pQE70, and only RNA) and positive controls (Fra a 1.06) were also used (b). In-gel RNase activity of the dephosphorylated Fra a 1.04, Fra a 1.05, Fra a 1.07 and Fra a 1.08 proteins (c). Proteins were dephosphorylated with calf intestinal alkaline phosphatase (CIP) and lambda phosphatase at 37 °C and 30 °C, respectively for an incubation time of 1 h. The polyacrylamide gels showed a clear region at 18 kDa corresponding to proteins with RNase activity.

services/NetPhos/) revealed six putative phosphorylation sites for the Fra a 1 proteins: three serine residues (Ser₁₂, Ser₈₅, and Ser₁₀₀), two threonine residues (Thr₅ and Thr₅₃) and one tyrosine residue (Tyr₆₇) (Fig. 1). Except for Fra a 1.06 (Thr₅₅), a Lys₅₅ residue was detected for the rest of Fra a 1 proteins suggesting that it could be important for phosphorylation (Fig. 1). Such post-translational modifications play regulatory roles in cellular function and gene expression of PR-10. Phosphorylation was revealed to be implicated or not in the RNase activity. In the hot pepper CaPR-10 protein, phosphorylation increased its ribonucleolytic activity to cleave invading viral RNAs and phosphorylation of PR-10 proteins may influence substrate specificity (Park et al., 2004). It was also suggested that increased amount of LRR1 (Leucine-rich repeat protein), which recognized pathogen attack and activated defence genes, enhanced both phosphorylation and RNase activity of the pepper PR-10 proteins Choi et al., 2012). Besides, in JcPR-10a nine putative phosphorylation sites were postulated which might regulate its activity (Agarwal et al., 2013). In the cocoa however, TcPR-10 phosphorylation had no influence on its RNase activity and both phosphorylated and non-phosphorylated TcPR-10 degraded plant RNA (Pungartnik et al. (2009) while in *Arachis hypogaea*, PR-10 proteins were established to be phosphorylated but their role in RNase activity was not shown (Jain et al., 2006).

As the recombinant proteins Fra a 1.04, Fra a 1.05, Fra a 1.07 and Fra a 1.08, which were produced in *E. coli*, were shown to be phosphorylated, it seemed that bacteria such as *E. coli* have exploited the

potential of protein phosphorylation. It was supposed for a long time that serine, threonine and tyrosine phosphorylation occurs only in Eukaryota (Bakal and Davies, 2000) whereas bacteria have histidine and aspartate kinases (Stock et al., 1990). Recently, the capacity of bacterial serine/threonine and tyrosine kinases to phosphorylate multiple substrates has been thoroughly investigated. Kinase phosphorylation sites were detected on many bacterial proteins such as those from *Bacillus subtilis* (Pietack et al., 2010; Ravikumar et al., 2014), *Mycobacterium tuberculosis* (Baer et al., 2014; Grundner et al., 2005) and *E. coli* (Grangeasse et al., 2003; Macek et al., 2008).

Altogether, the issue of how and where Fra a 1 proteins are phosphorylated is challenging and remains ambiguous. Since this report is the first work proving the phosphorylation of Fra a 1 proteins, the determination of the 3D structure of the Fra a 1 proteins could help to get insight into such intriguing questions.

3.6. Protein dephosphorylation and Ribonuclease activity assay

3.6.1. In-solution RNase activity

Assuming that Fra a 1.04, Fra a 1.05, Fra a 1.07 and Fra a 1.08 proteins are phosphorylated and that only Fra a 1.06 possessed RNase activity, the question arose whether phosphorylation was influencing the RNase activity. It has already been shown that non-phosphorylated CaPR-10 also possess RNase activity but this activity was much lower compared to the phosphorylated form (Park et al., 2004). Thus, the

recombinant Fra a 1 proteins were dephosphorylated by two phosphatases and the effect on their RNase activity was addressed. Alkaline phosphatase calf intestinal (CIP) treatment was performed at 37 °C, while lambda phosphatase hydrolysis was conducted at 30 °C, both of them for 1 h and overnight (Fig. 5). The in-solution RNase activity was tested by incubating the four recombinant proteins in the presence of total RNA isolated from strawberry at pH 7.4. Under these conditions, RNA was degraded by the four dephosphorylated proteins after 4 h (Fig. 5). Concerning the dephosphorylation efficiency of CIP and lambda phosphatase, no significant difference was found for both enzymes. In addition, controls were treated with CIP and in-solution RNase activity was tested (Fig. 5). No degradation was detected when RNA was incubated alone, only with the empty vector (pQE70), with the buffer or with H_2O . Fra a 1.06, which was used as a positive control, was corroborating the efficiency of this assay.

3.6.2. In-gel RNase activity

To test the in-gel RNase activity, dephosphorylated Fra a 1 proteins were separated on 12% acrylamide gel containing 2.4 mg/mL total RNA of torula yeast. After electrophoresis, the gel was stained with toluidine blue and destained with Tris-HCl. The gel was incubated 50 min at 51 °C to allow the RNase to digest the RNA. The obtained results (Fig. 5) clearly proved that the four recombinant proteins, dephosphorylated with CIP and lambda phosphatase exhibited ribonucleolytic activity against total RNA, strengthening the results of the in-solution RNase activity (Supplementary material Figure S8). Using lambda phosphatase, the white bands at 18 kDa indicating RNase activity were weak for Fra a 1.07 and Fra a 1.08 after dephosphorylation for 1 h but visible after overnight dephosphorylation whereas after CIP treatment RNase activity was observable after 1 h and overnight treatments. Dephosphorylation depends on the phosphatase and its concentration as well as on sample preparation including buffer composition. An *in vitro* dephosphorylation analysis of the cardiac muscle proteome using different phosphatases (CIP, lambda phosphatase and protein phosphatases PP1 and PP2A) showed that, compared to PP1 and PP2A, CIP and lambda phosphatase displayed reduced substrate specificity (Husberg et al., 2012).

4. Conclusion

Based on information provided by the *Fragaria vesca* genome, putative PR10-related Fra a 1 isoforms were identified in *Fragaria* × *ananassa*, and selected recombinant proteins were functionally characterized. Expression analyses revealed significant transcript levels in roots and fruits consistent with their assumed function in plant defence. The plant proteins, heterologously expressed in *E. coli* show a phosphorylation dependent RNase activity. The frequent presence of phosphorylation sites in PR-10 proteins suggest that these proteins are regulated and activated by similar mechanisms. Altogether, the findings of this work increase our knowledge on the biological significance of the strawberry Fra a 1 genes, which is a key to understand the functions of PR-10 proteins.

One sentence summary

Analysis of Fra a 1 isoforms in *Fragaria* × *ananassa* strawberry plants revealed that they are expressed differently, that they can be phosphorylated and hydrolyze RNA.

Notes

Declarations of interest: none.

During the completion of the manuscript, a new version of the *F. vesca* genome (v 4.0) was published and new gene names were introduced (Edger et al., 2017; <https://www.rosaceae.org/>). Readers are asked to refer to Supplementary material Table S1 for gene names

comparison.

Author contributions

F.B. performed the experiments and wrote the manuscript, K.F.O. cloned Fra a 1.01-03 genes and W.S. planned the experiments, supervised the study and corrected the manuscript.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.jplph.2018.12.002>.

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Supplemental material

Phosphorylation-dependent ribonuclease activity of Fra a 1 proteins

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Supplemental material Table S1. Primers of the 21 *Fra a 1* isoforms used for qPCR analysis.

Supplemental material Table S2. Primers used for the cloning of *Fra a 1.01-08*.

Supplemental material Table S3. Nomenclature of *Fra a 1.01-08* isoforms in *F. × ananassa* and their orthologues in the *F. vesca* genome.

Supplemental material Table S4. Pairwise protein sequences similarity of the eight *Fra a 1.01-1.08* proteins.

Supplemental material Figure S1. SDS-PAGE analysis of the five purified *Fra a 1.04-1.08* proteins

Supplemental material Figure S2. Protein gel blot analysis of the five purified *Fra a 1.04-1.08* proteins.

Supplemental material Figure S3. Multiple alignment of amino acid sequences of Bet v 1 and 21 similar sequences from *F. × ananassa* and *F. vesca*.

Supplemental material Figure S4. Phylogenetic tree of *Fra a 1* genes and summary of the qPCR analysis of the 21 *Fra a 1* isoforms.

Supplemental material Figure S5. Protein gel blot analysis using a phosphoserine/ -threonine/ -tyrosine antibody for the detection of phosphorylated *Fra a 1.04-1.08* proteins.

Supplemental material Figure S6. Phosphoprotein detection of *Fra a 1.04-1.08* proteins using Pro-Q Diamond staining gel

Supplemental material Figure S7. Chemiluminescent assay of *Fra a 1.04-1.08*

Supplemental material Figure S8. In-gel RNase activity of the dephosphorylated *Fra a 1.04*, *Fra a 1.05*, *Fra a 1.07* and *Fra a 1.08* proteins.

Table S1. Primers of the 21 *Fra a 1* isoforms used for qPCR analysis and new gene numbers according to *F. vesca* genome sequence (v 4.0) (<https://www.rosaceae.org/>).

Primer name	Primer sequence (5'→3')	Gene numbers (v 4.0)
<i>Fra a 1.04_f</i> <i>Fra a 1.04_r</i>	AGCTCTTCTTTTCTTGTCACCTT GGTTCCTACGCCTCCATCTC	FvH4_4g19710.1
<i>Fra a 1.05_f</i> <i>Fra a 1.05_r</i>	TCCTTCAACTTCTCAAACACCA GTTCTACGCCTCCGTCTC	FvH4_4g19060.1.a
<i>Fra a 1.06_f</i> <i>Fra a 1.06_r</i>	TTTTATCATTTCCAGTTTGTAAGC CCTTCAACGATTTACACACA	FvH4_4g19080.1
<i>Fra a 1.07_f</i> <i>Fra a 1.07_r</i>	TCAGTCTTCTCATCTTAAGCTTGC GTTCCAACACCTCCATCACC	FvH4_4g19700.1
<i>Fra a 1.08_f</i> <i>Fra a 1.08_r</i>	TTTCAATCTTCCCATTTTTAGCTC GTATTCGCTCCCTTCACCAA	FvH4_4g18850.1
<i>Fra a 1.01_f</i> <i>Fra a 1.01_r</i>	TCCTTATTCCTGCTTGCTT AGGTGATCTTCTTGATGGTTCC	FvH4_4g18990.1
<i>Fra a 1.02_f</i> <i>Fra a 1.02_r</i>	CGTGGA GATCAAGGAAGAGC GGAACATCAGCGGAACAAAT	FvH4_4g19060.1.b
<i>Fra a 1.03_f</i> <i>Fra a 1.03_r</i>	GTAAAGGCCGAAAGGAAAG GATCGAGCAGATTGACACCA	FvH4_4g19010.1
04962_f 04962_r	CTTGTCATTTCCCAACTCA ATGGTTTCAGCACCTTGAC	FvH4_4g19120.1
07083_f 07083_r	TTCCCTTCAGCTCTTCTACATTG GAACGATTTACGCTCTTC	FvH4_4g19020.1
07077_f 07077_r	TCAGAAGCTAGCTGAGCCAAG TCACCTCCAGGAATTGCAT	FvH4_4g18960.1a
32299_f 32299_r	CCGTTTACCTCATCAAATTATTAAGTC TTCCCCGAGGTTGATCTTCT	FvH4_5g00780.1
05185_f 05185_r	TGTCTTCAAGTACGAGGCTGAA CAATGGCATCAATTCTGTGC	FvH4_4g19170.1
07063_f 07063_r	TCAACCTCATCAGTCACACCA CTTCGCCAAAGGTGATCTTC	FvH4_4g18830.1
07076_f 07076_r	GCACAGAATCGATGAACTCG GCTTCTCCAATGGGGTTGTA	FvH4_4g18960.1b
07085_f 07085_r	TTCTCTGATCATTTCCCTTTTAGC CCAACTCCTCCATCACCTTC	FvH4_4g19050.1
07081_f 07081_r	CGAATTGAGAATTTTCGATCCTA TACTGCCTTCCCCAAAGGTA	FvH4_4g19000.1
11094_f 11094_r	TCTGGTCAATTTATCAGCAA TAATGGCCTGAGGCATGAGT	FvH4_2g26160.1
07084_f 07084_r	TGGGTCTGTTCTTTGATCAATTT TCAGCATCGAGAACAAATGC	FvH4_4g19040.1
07087_f 07087_r	TCACCTTATGGAGACCTAGCTATTG GCTGGGGTGCTTCTTTGAC	FvH4_4g19070.1
07078_f 07078_r	TTTGAGGTGATTAACACATAGATTACG ACCGCACTAGCCATGAATGT	FvH4_4g18970.1
Interspacer_f Interspacer_r	ACCGTTGATTTCGCACAATTGGTCATCG TACTGCGGGTCGGCAATCGGACG	

Table S2. Primers used for the cloning of *Fra a 1.01-08*.

Primer name	Primer sequence (5'→3')
<i>Fra a 1.04_f</i>	GATCGCATGCATGGGTGTCTTCACA
<i>Fra a 1.04_r</i>	GATCGGATCCTTAACAGTATTCATT
<i>Fra a 1.05_f</i>	GATCGCATGCATGGGTGTATTCACT
<i>Fra a 1.05_r</i>	GATCGCATGCTTAACAGTATTCATT
<i>Fra a 1.06_f</i>	GATCGCATGCATGGGTGTGTTCACT
<i>Fra a 1.06_r</i>	GATCGGATCCACAGTATTCATTAGGATT
<i>Fra a 1.07_f</i>	GATCGCATGCTGGGCGTGTTCACTTAT
<i>Fra a 1.07_r</i>	GATCGGATCCGTTGTATGCATCAGGGTT
<i>Fra a 1.08_f</i>	GATCGCATGCTGGGTGTGTTACATAT
<i>Fra a 1.08_r</i>	GATCGGATCCGTTGTAGGCCTCAGGGTT
<i>Fra a 1.01_f</i>	GATCGCATGCTGATGGGTGTTTACACTTATGAAAACGAG
<i>Fra a 1.01_r</i>	GATCGGATCCGTTGTATTCGCTGGGGTGGTCCTTGAG
<i>Fra a 1.02_f</i>	GATCGCATGCTGATGGGTGTGTTCACTTATGAAACCGAG
<i>Fra a 1.02_r</i>	GATCGCATGCAACAGTATTCATTAGGATTGGCCAAGAG
<i>Fra a 1.03_f</i>	GATCGCATGCTGATGGGTGTGTTACATACGAATCCGAG
<i>Fra a 1.03_r</i>	GATCGGATCCGTTGTATTCCTCAGGATGGGCCAAGAGG

Table S3. Nomenclature of *Fra a 1.01-08* isoforms in *F. × ananassa* and their orthologues in the *F. vesca* genome.

Gene name in <i>F. × ananassa</i>	Gene name in <i>F. vesca</i>	GenBank accession no.	ORF (nt)	AA length	MW (kDa)	Pairwise identity related to Bet v 1 (%)
<i>Fra a 1.01</i>	<i>FvH4_4g18990.1</i>	AM236319	483	160	17.76	53.1
<i>Fra a 1.02</i>	<i>FvH4_4g19060.1.b</i>	GQ148818	483	160	17.49	58.0
<i>Fra a 1.03</i>	<i>FvH4_4g19010.1</i>	GQ148819	480	159	17.44	58.4
<i>Fra a 1.04</i>	<i>FvH4_4g19710.1</i>	KJ507735	483	160	17.51	55.6
<i>Fra a 1.05</i>	<i>FvH4_4g19060.1.a</i>	KJ507736	483	160	17.48	57.4
<i>Fra a 1.06</i>	<i>FvH4_4g19080.1</i>	KJ507737	483	160	17.47	56.2
<i>Fra a 1.07</i>	<i>FvH4_4g19700.1</i>	KJ507738	483	160	17.58	59.3
<i>Fra a 1.08</i>	<i>FvH4_4g18850.1</i>	KJ507739	483	160	17.52	58.6

Table S4. Pairwise protein sequences similarity of the eight *Fra a 1.01-1.08* proteins. Percentage of sequence similarities higher than 90% are highlighted in red. *Fra a 1.02* and *Fra a 1.05* showed a 99.4% of similarity. Protein sequence similarities were performed using Geneious software.

	<i>Fra a 1.02</i>	<i>Fra a 1.03</i>	<i>Fra a 1.04</i>	<i>Fra a 1.05</i>	<i>Fra a 1.06</i>	<i>Fra a 1.07</i>	<i>Fra a 1.08</i>
<i>Fra a 1.01</i>	79.4	75.6	76.3	78.8	76.3	69.4	70.6
<i>Fra a 1.02</i>		80.6	95.6	99.4	96.3	75.6	77.5
<i>Fra a 1.03</i>			78.1	80.0	78.8	88.1	90.6
<i>Fra a 1.04</i>				96.3	97.5	73.8	75.6
<i>Fra a 1.05</i>					96.9	75.0	76.9
<i>Fra a 1.06</i>						75.0	76.9
<i>Fra a 1.07</i>							96.3

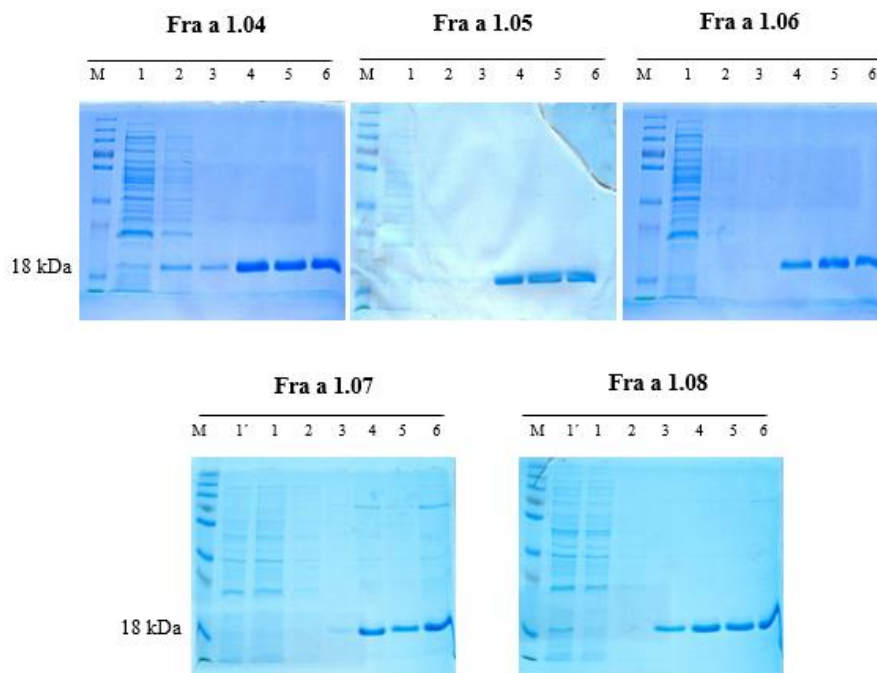


Figure S1. SDS-PAGE analysis of the five purified Fra a 1.04-1.08 proteins (10 µg); Lane M: molecular weight marker; Lane 1': Crude extract, Lane 1: Flow through; Lane 2, 3: Washes; Lane 4, 5, 6: Elution fractions. Bands were visualized by Coomassie Brilliant Blue G250. A band of 18 kDa was revealed for the five Fra a 1 proteins.

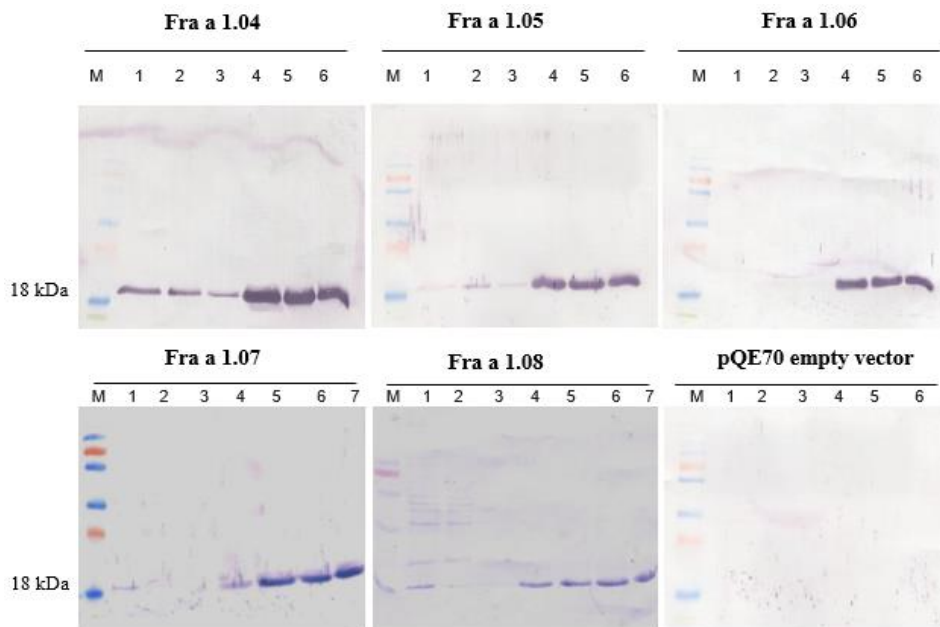


Figure S2. Protein gel blot analysis of the five purified Fra a 1.04-1.08 proteins (10 µg); Lane M: molecular weight marker; Lane 1: Flow through; Lane 2, 3: Washes; Lane 4, 5, 6, 7: Elution fractions. Bands were visualized on polyvinylidene fluoride (PVDF) membrane with monoclonal anti-6× His AP antibody.

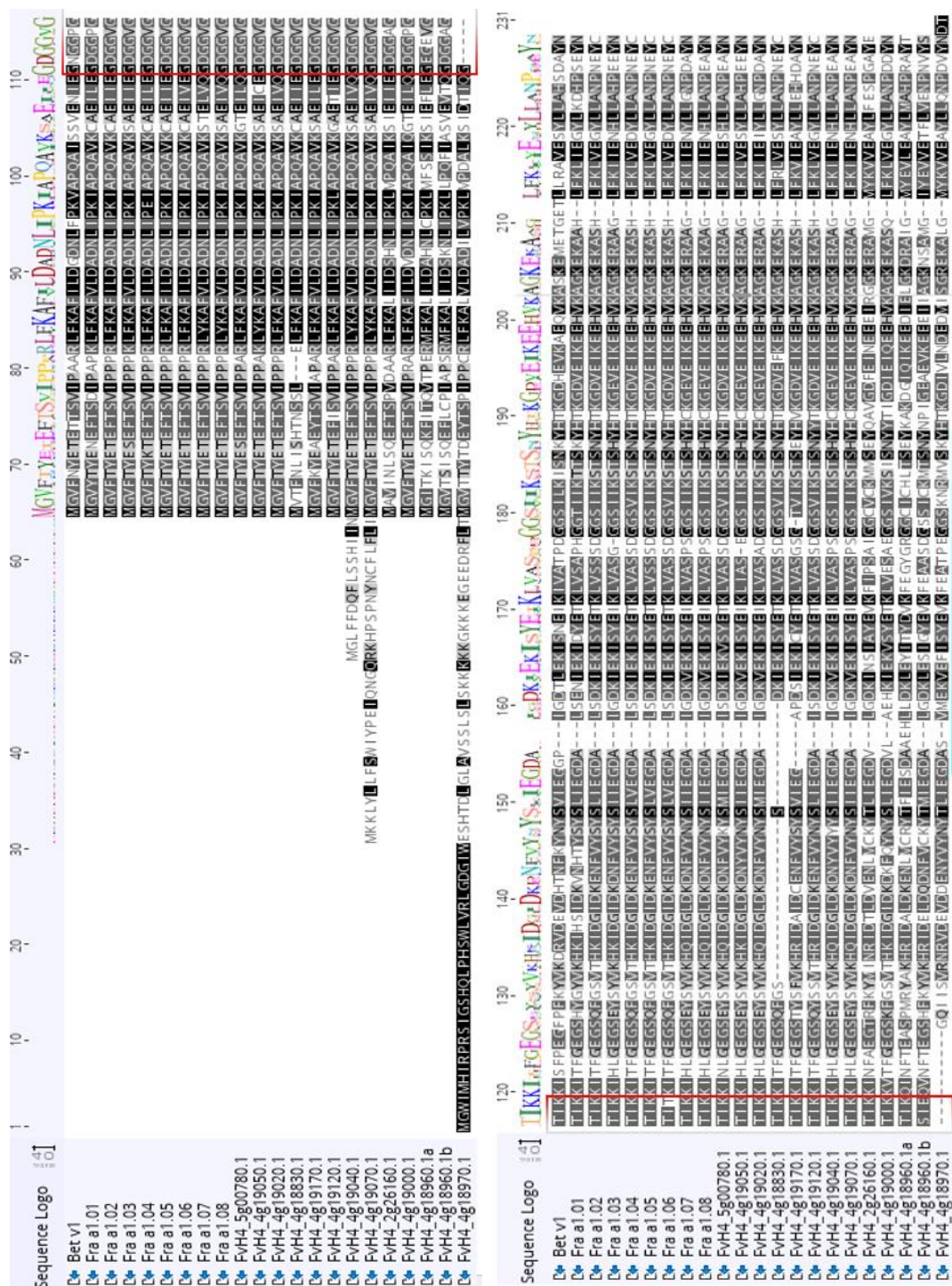


Figure S3. Multiple alignment of amino acid sequences of Bet v 1 and 21 similar sequences from *F. × ananassa* and *F. vesca*. The P-loop motif (GxGxGxxK) is highlighted by a red box and blue underline indicate the Bet v 1 motif: G-D-A-[L/I]-[S/G]-D-K-[I/V]-E-K-I-S-Y-E-[T/I]-K-L-V-[S/A]-S-[S/P]-[D/S]-G-G-S-[V/I]-I-K-S-T-S-[N/H]-Y. Alignment was performed with Geneious software.

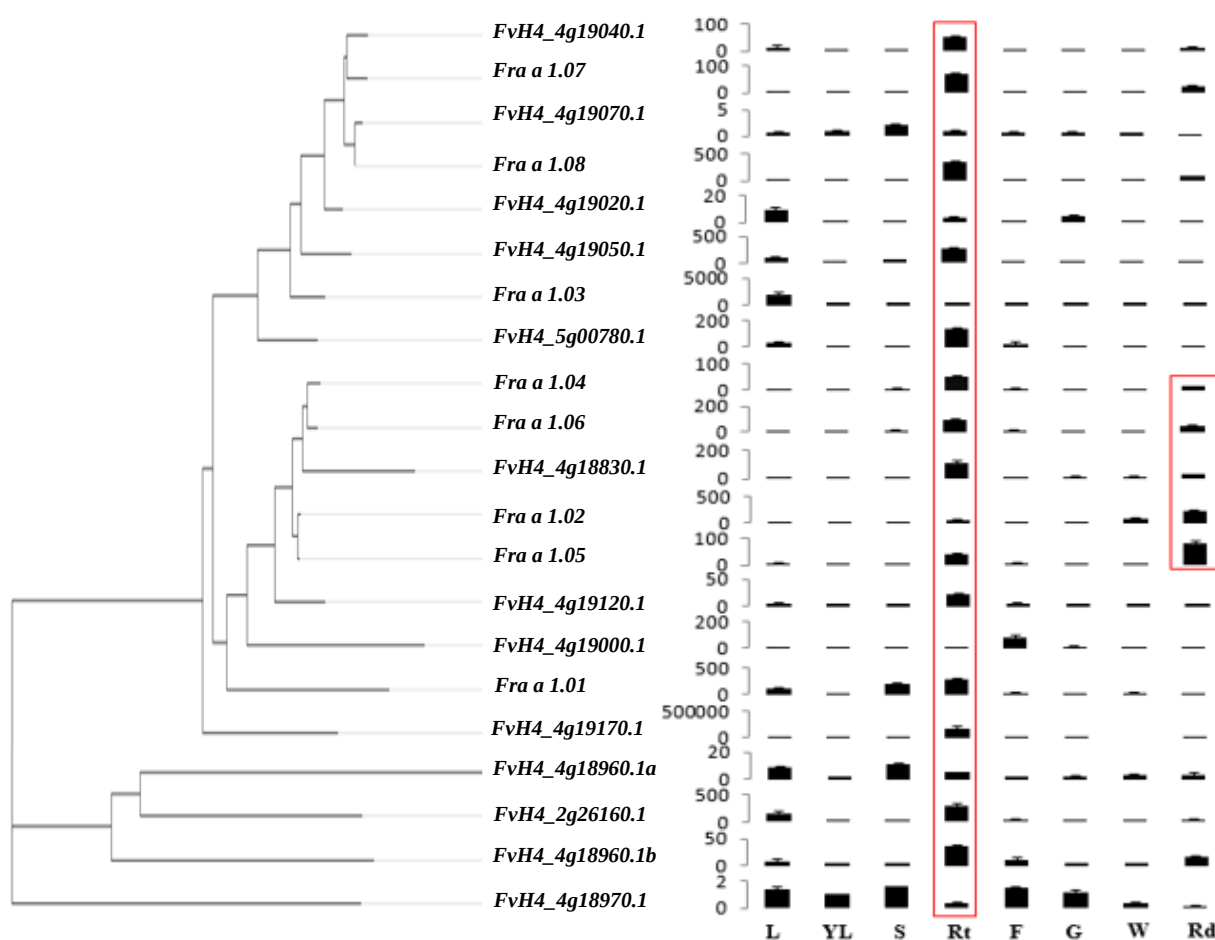


Figure S4. Phylogenetic tree of *Fra a 1* genes and summary of the qPCR analysis of the 21 *Fra a 1* isoforms in leaf (L), young leaf (YL), stem (S), root (Rt), flower (F), green (G), white (W) and red (Rd) fruit. Expression analysis was monitored by qPCR using specific primers for *Fra a 1* genes and the *FaRIB413* gene as reference gene.

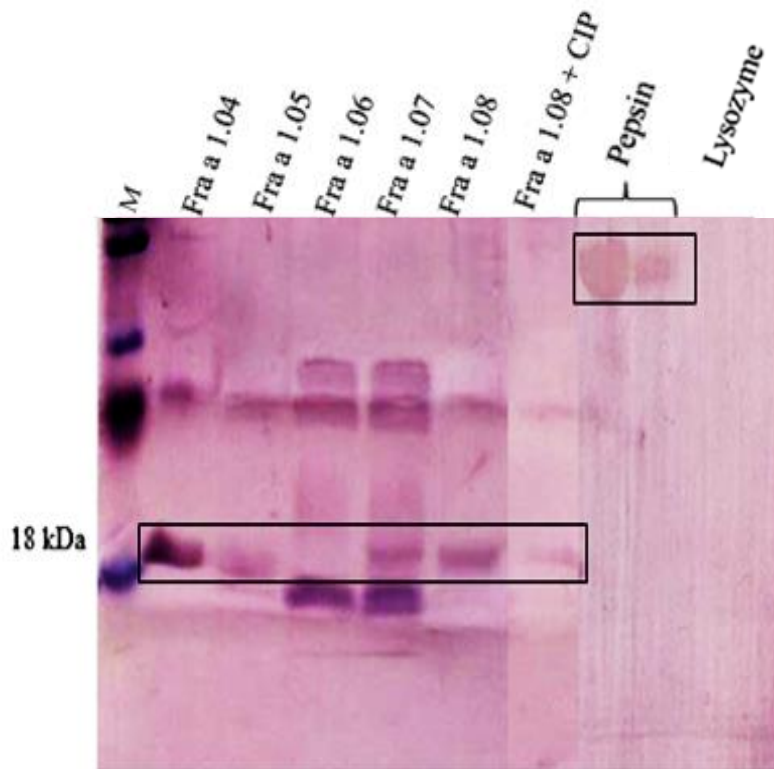


Figure S5. Protein gel blot analysis using a phosphoserine/ -threonine/ -tyrosine antibody for the detection of phosphorylated Fra a 1.04-1.08 proteins. Positive (pepsin, 43 kDa) and negative (lysozyme, 14.3 kDa) controls were also used. Fra a 1.04, 1.05, 1.07 and 1.08 were shown to be phosphorylated due to the bands at 18 kDa. M: molecular weight marker.

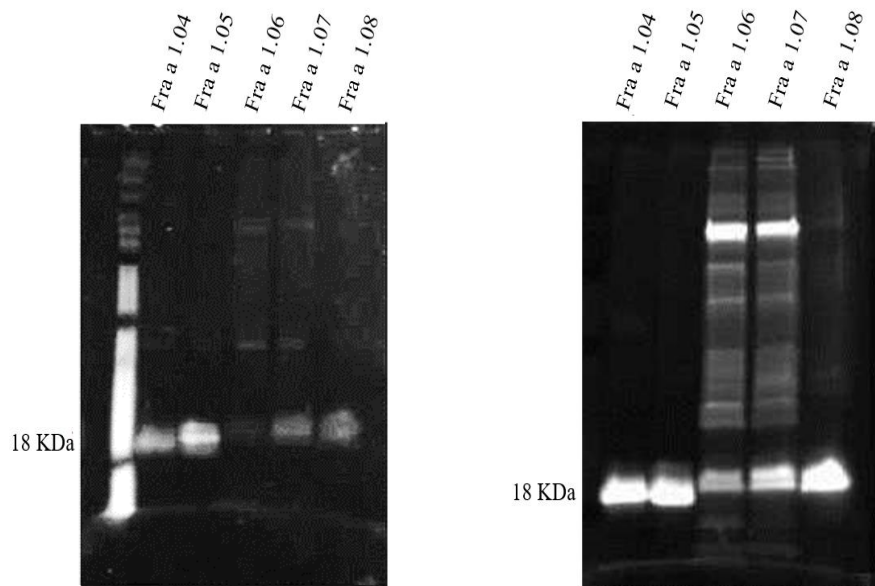


Figure S6. Phosphoprotein detection of Fra a 1.04-1.08 proteins using Pro-Q Diamond staining gel (left). Gel shown on the right was subsequently stained for total proteins detection with Sypro Ruby stain. Only Fra a 1.04, Fra a 1.05, Fra a 1.07 and Fra a 1.08 were demonstrated to be phosphorylated (left).

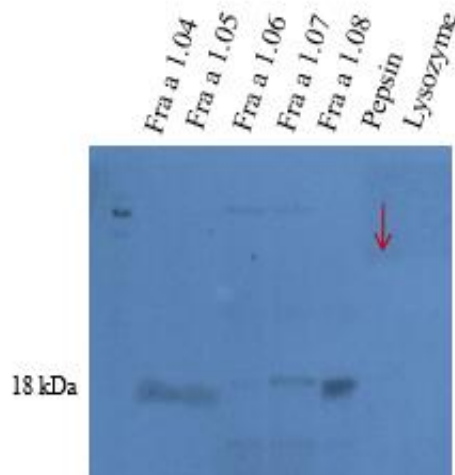


Figure S7. Chemiluminescent assay of Fra a 1.04-1.08 (10 µg). Horseradish Peroxidase-conjugated antibody and an enhanced luminol-based chemiluminescent substrate were used for the chemiluminescent protein blotting detection. Exposure to X-ray film indicated that Fra a 1.04, Fra a 1.05, Fra a 1.07 and Fra a 1.08 are phosphorylated. Positive (pepsin, 43 kDa) and negative controls (lysozyme, 14.3 kDa) were also used.

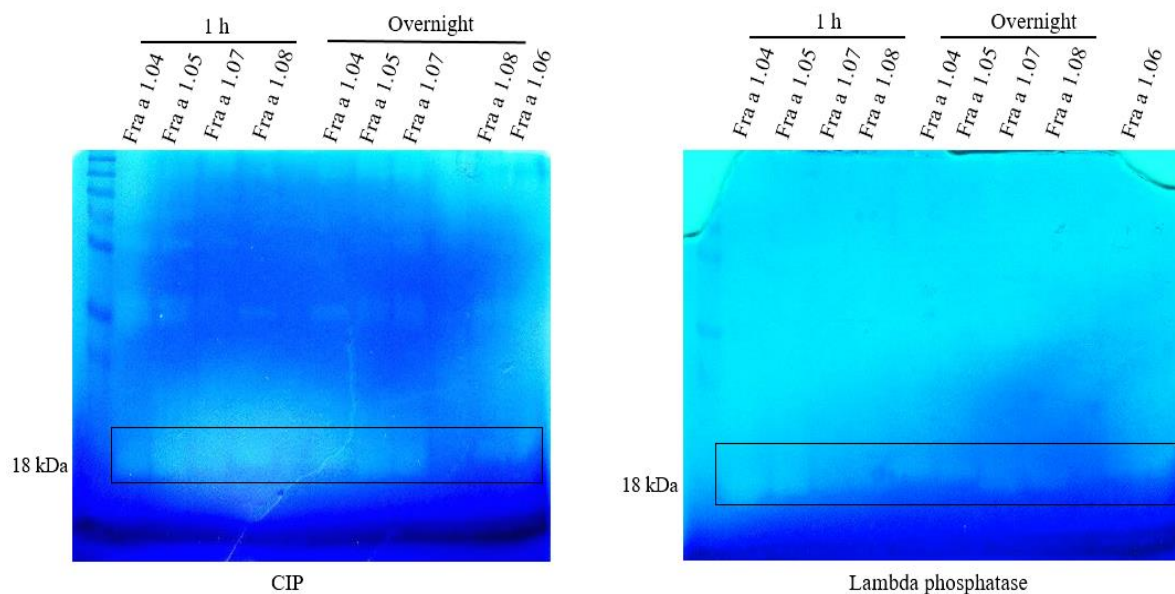


Figure S8. In-gel RNase activity of the dephosphorylated Fra a 1.04, Fra a 1.05, Fra a 1.07 and Fra a 1.08 proteins. Proteins were dephosphorylated with calf intestinal alkaline phosphatase (CIP) and lambda phosphatase at 37 °C and 30 °C, respectively for an incubation time of 1 h and overnight. The polyacrylamide gels showed a clear region at 18 kDa corresponding to proteins with RNase activity. Fra a 1.06 was used as positive control.