

Wilfried Schwab **Physical Interaction between the Strawberry Allergen Fra a 1 and an Associated Partner FaAP***

Interaction of Fra a 1 proteins and FaAP

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ABSTRACT

The strawberry fruit allergens Fra a 1.01E, Fra a 1.02 and Fra a 1.03 belong to the group of pathogenesis-related 10 (PR-10) proteins and are homologues of the major birch pollen Bet v 1 and apple allergen Mal d 1. Bet v 1 related proteins are the most extensively studied allergens but their physiological function *in planta* remains elusive. Since Mal d 1-Associated Protein (MdAP) has been previously identified as interaction partner of Mal d 1 we studied the binding of the orthologous Fra a 1-Associated Protein (FaAP) to Fra a 1.01E/1.02/1.03. As the C-terminal sequence of FaAP showed strong auto-activation activity in yeast two-hybrid analysis a novel time resolved DNA-switching system was successfully applied. Fra a 1.01E, Fra a 1.02 and Fra a 1.03 bind to FaAP with K_D of 4.5 ± 1.1 nM, 15 ± 3 nM, and 11 ± 2 nM, respectively. Fra a 1.01E forms a dimer, whereas Fra a 1.02 and Fra a 1.03 bind as monomer. The results imply that PR-10 proteins might be integrated into a protein-interaction network and FaAP binding appears to be essential for the physiological function of the Fra a 1 proteins.

INTRODUCTION

Plant Pathogenesis-Related class 10 proteins (PR-10) are part of the highly complex plant inducible defence mechanism¹ and are expressed upon biotic (pathogen infection) and abiotic (wounding, salt, heat) stress factors.² The PR-10 protein family comprises small (17-22 kDa), cytosolically localised, and slightly acidic proteins that are widely distributed among seed plants. PR-10 genes constitute a multigene family in which some members are constitutively expressed in defined plant tissues while others are induced under certain environmental conditions.^{3,4} This gives rise to the assumption that PR-10 proteins play a role in plant development as well as in the general plant defence mechanism.⁵ Till now the

physiological function of PR-10 proteins in plants has remained uncharacterized although some members in this protein family show ribonuclease activity and are discussed as plant steroid carrier.⁶ PR-10 proteins are homologues of a large family of food and tree pollen allergens¹ – a subdivision of the Bet v 1 superfamily.⁵ Prominent PR-10 protein members that induce allergenic reactions in human are Bet v 1 from birch pollen⁷, Mal d 1 from apple⁸, Pru ar 1 from apricot, Pru av 1 from cherry⁹, Dau c 1 from carrot¹⁰, Api g 1 from celery¹¹ and the strawberry (*Fragaria × ananassa*) fruit allergen Fra a 1.¹²

Cloning and sequencing of *Fra a 1* genes in the cultivated strawberry (*Fragaria × ananassa*) revealed the presence of an intron and little variability in the encoded amino acid sequence (Fra a 1 isoforms A to E).¹³ However, the recent identification of novel *Fra a 1* genes and the complete genome sequence of the related species *F. vesca* show that the genus *Fragaria* contains a family of 13-21 *Fra a 1* homologous genes, depending of the selection criterion¹⁴⁻¹⁶, and *Fra a 1A – 1E* are probably allelic forms of *Fra a 1.01*. Although Fra a 1.01E has been proposed as the major strawberry allergen^{13,14}, gene expression analysis and basophil activation tests revealed Fra a 1.02 as the main strawberry allergen of the Bet v 1 superfamily.¹⁷

The recently elucidated solution structure of Fra a 1.01E exhibits a high similarity to the crystal structure of the major cherry allergen Pru av 1 and the major birch pollen allergen Bet v 1 (sequence identity of 79.4% and 53.8%, respectively) and a glycine-rich loop motif (residues 46-51) was verified as a highly conserved feature throughout the Bet v 1 superfamily.¹⁸

Fra a 1.01E/1.02/1.03 proteins have a high sequence similarity. The pairwise identity of the amino acid sequence of Fra a 1.01E and Fra a 1.03 with Fra a 1.02 is 78.9% and 80.1%, respectively. The sequence identity of the three isoforms relating to Bet v 1 accounts for 54%, 58.4% and 59%, respectively.¹⁷

Fra a 1.01E is mainly expressed in roots, *Fra a 1.02* in ripe strawberry fruits and *Fra a 1.03* shows similar expression levels in different tissues and developmental stages.^{15,18}

Transient RNAi-mediated silencing of *Fra a 1* genes in strawberry fruit of the red-fruited cultivar Elsanta led to reduced levels of *Fra a 1* mRNA in the fruits and significantly decreased levels of anthocyanins and upstream metabolites.^{14,15,19} This effect was consistent with the parallel down-regulation of phenylalanine ammonia lyase (*FaPAL*) and to a lesser extent of chalcone synthase (*FaCHS*) transcript levels. Both genes have been shown to be involved in the production of anthocyanins in fruits. These results demonstrated that *Fra a 1* expression is directly linked to flavonoid biosynthesis and showed that the *Fra a 1* allergen has an essential biological function in pigment formation in strawberry fruits.¹⁵ Only recently, the structural analysis of a *Fra a 1.03*-catechin complex provided first mechanistic insights into the biological function of *Fra a 1* proteins as they may play a role in the control of the flavonoid pathway by binding to metabolic intermediates.²⁰

In a yeast two-hybrid screen (Y2H) to reveal proteins that interact with the major apple allergen Mal d 1, Mal d 1-Associated Protein (MdAP) was identified.²¹ MdAP comprises 190 amino acids and does not exhibit any sequence similarity to known proteins but shares 69% and 46% amino acid identity with an orthologous protein in *F. vesca*¹⁶ which we named *Fra a 1*-Associated Protein (FaAP) and a protein in *Arabidopsis thaliana*, respectively. MdAP and FaAP are encoded by a single gene in the *M. × domestica* and *F. vesca* genome.

Powerful methods for the physical-chemical characterization of proteins and their physical interactions are highly sought after as they are important tools for functional analysis of proteins. *In vitro* molecular interactions are commonly analysed with biosensors, which determine the association/dissociation of solute target proteins to/from surface-immobilized capture molecules. The switchSENSE technique is such a biosensor, which was developed to

investigate the molecular dynamics of proteins.²² Briefly, short doubled stranded 48-x DNA nanolevers [Fig. 1] are end-tethered to gold microelectrodes that are arranged within individually addressable flow channels. A covalently bound fluorescence dye which is attached to the opposite end of the DNA is used to measure the orientation of the DNA levers with respect to the surface. Alternating potentials that are applied to the electrodes switch the lever orientation between a standing and a lying state. As the fluorescence signal is quenched when the DNA levers tilt towards the gold surface the movement of the DNA levers can be monitored by an epifluorescence setup in real-time. When designated capture molecules are attached to the DNAs' top end, they can specifically bind target proteins from solution. As a consequence of the increased hydrodynamic drag, the motion of the DNA lever slows down as a function of the protein's hydrodynamic diameter. Protein-protein interactions slow down the motion further. This allows following of binding kinetics in real time and calculation of association k_{on} and dissociation rates k_{off} , and the dissociation constant K_D .

The aim of this study was to test the interaction of three Fra a 1 protein isoforms with the putative binding partner FaAP by *in vivo* and *in vitro* methods. The well-established yeast two-hybrid assay was used to study *in vivo* protein binding of the target proteins whereas the switchSENSE method was applied for *in vitro* analysis.²² The latter system takes advantage of short DNA strands that function as nanolevers and are employed as capture probes to bind target proteins. By moving the proteins over nanometer amplitudes the protein diameter were determined and dissociation constants (K_D) calculated.²²

MATERIAL AND METHODS

RNA extraction and cloning of Fra a 1.01E, Fra a 1.02, Fra a 1.03 and FaAP for heterologous expression

Total RNA was extracted from strawberry fruit (*Fragaria × ananassa* cv. Elsanta) as described previously.²³ First-strand cDNA was synthesized as reported.²⁴ The corresponding ORF sequences of three isoforms of *Fra a 1* (*Fra a 1.01E/1.02/1.03*) were PCR amplified using strawberry cDNA as template and digested with *SphI* and *BamHI* (*Fra a 1.02: SphI*) for the vector pQE70 including a His-tag (Qiagen, Hilden, Germany). The resulting digested PCR fragments were ligated into the *SphI/BamHI* (*Fra a 1.02: SphI*) predigested pQE70 vector. The clones containing the recombinant plasmids pQE70-r*Fra a 1.01E/1.02/1.03* were screened by PCR, restriction digestion, and verified by sequencing, to confirm correct insertion of the genes *rFra a 1.01E/1.02/1.03* in frame with the C-terminal His-tag. The validated constructs pQE70-r*Fra a 1.01E/1.02/1.03* were transformed in *E. coli* strain BL21 (DE3) pLysS (Novagen, Darmstadt, Germany). The gene of the putative interaction partner *FaAP* was cloned as described for the *Fra a 1* isoforms. *FaAP* was fused N-terminal to EGFP (Enhanced Green Fluorescent Protein), separated by a cleavage site for thrombin protease that was inserted as linker. The appropriate restriction enzymes are *BamHI* and *EcoRI*. The *FaAP*-EGFP fusion construct was cloned in the vector pET28b including an N- and C-terminal His-tag in frame with *FaAP*-EGFP. *FaAP* was expressed as the fusion protein *FaAP*-EGFP and used in the experiments, as unmodified *FaAP* was barely expressed by *E. coli*.

Heterologous expression, purification of recombinant *Fra a 1* and *FaAP* proteins

Heterologous expression was performed as described.²⁴ The proteins were purified according to²⁴ and the manual of ProfinityTM IMAC Resins.²⁵ GenBank Accession Numbers are AM236319 (*Fra a 1.01E*), GQ148818 (*Fra a 1.02*), GQ148819 (*Fra a 1.03*) and KF785812 (*FaAP*).

***Fra a 1.02*-specific rabbit polyclonal antibody**

The rabbit polyclonal antibody anti-Fra a 1.02 was raised against purified recombinant Fra a 1.02 protein according to ¹⁷.

Yeast Two-Hybrid analysis

A Y2H screen was performed as described previously.²⁶ The MATCHMAKER Two-Hybrid System 3 (Clontech) was used to identify protein-protein interactions. The corresponding ORF sequences of three strawberry allergen isoforms *Fra a 1.01E/1.02/1.03*, *Mal d 1* (cDNA from the cultivar “Golden Delicious”; GenBank Accession Number AJ417551) and *MdAP* (cDNA from the cultivar “Golden Delicious”; GenBank Accession Number AAD21467) ²¹ were cloned in GBD as well as in GAD. The *Saccharomyces cerevisiae* strain Y190 (Clontech) was used for the transformation using the *S. cerevisiae* EasyComp™ Transformation Kit (Invitrogen; Catalog no. K5050-01). Transformed Y190 yeast cells were plated on minimal medium lacking tryptophan (W) and leucine (L). To examine Y2H interaction of GAD- and GBD-fused proteins in the yeast cells, the transformants were grown for 4 d at 30°C on a minimal medium lacking W, L and histidine (H) and in addition of 2.5 to 7.5 mM (max. 20 mM) 3-amino-1,2,4-triazole (3-AT; Sigma-Aldrich). For negative controls the empty “bait” respectively “prey” vector was co-transformed with each putative binding partner. As positive control AMSH3 (“prey”) and VPS2.1 (“bait”) were analyzed according to ²⁷. The results were documented by photography. β -Galactosidase filter assay was performed as described in the “Yeast Protocols Handbook” PT3024-1 (Clontech).

Biochip and DNA layer preparation

Experiments on switchable DNA layers were performed on a biochip as described elsewhere.²² Briefly, thiolated 48-x oligonucleotides (Metabion, Planegg, Germany) were end-grafted to Au electrodes (120 μ m diameter) on a glass substrate via spotting with a

picoliter dispensing system [Fig. 1]. After 10 min incubation under co-adsorption of mercaptohexanol for surface passivation, the biochip was assembled and flow channels were formed by using double adhesive film as an intermediate layer and an ITO (indium-tin-oxide) cover slide as a top layer and counter electrode. Double-stranded DNA layers were denatured by rinsing NaOH solution (pH 12–13) over the electrodes for 1 s, after which layers could be regenerated by hybridization with fresh complementary DNA.

Modification of DNA with capture molecules and proteins

Nitrilotriacetic acid (NTA₃)-tagged 48-x DNA was synthesized from triaminated DNA (IDT, Leuven, Belgium).^{28,29} The NTA chelator heads were loaded by 10 min incubation in 500 μ M NiCl₂ solution after removal of excess divalent ions with 5 mM EDTA. Protein-DNA conjugates of 48-x DNA with Fra a 1.01E, Fra a 1.02 and Fra a 1.03 (500 nM Fra a 1 protein) were synthesized using a SMCC-Linker (Succinimidyl-4-[N-maleimido-methyl]cyclohexane-1-carboxylate) as described elsewhere²² without further purification.

Measurement of the protein-protein interaction between RCAR8 and ABI2 with and without abscisic acid (control experiment)

His-tagged ABI2 (*Abscisic Acid Insensitive 2*) was bound to NTA₃-tagged 48-x DNA in T-buffer at a concentration of 50 nM. The interaction with RCAR8 ((PYL5), *Regulatory Components of ABA Receptor 8 (Pyrabactin Resistance 1-Like 5)*) and ABA (abscisic acid) was investigated by subsequent rinsing of 100 nM RCAR8 with and without 10 μ M abscisic acid over the immobilized ABI2 in changing order. As negative control, all experiments were repeated with unmodified 48-x DNA.

Measurement of the protein-protein interaction between Fra a 1 and FaAP-EGFP

The respective proteins were solved in His-tag elution buffer. The His-tag elution buffer was changed via ZebaTM desalt spin columns (Thermo Scientific, Rockford, USA) against T-buffer (10 mM Tris-HCl pH 7.3, 50 mM NaCl). FaAP-EGFP and Fra a 1.02 antibody were bound to DNA layers of DNA-Fra a 1.01E, -Fra a 1.02 and -Fra a 1.03 conjugates by flowing a solution of 200 nM or 500 nM FaAP-EGFP, respectively 50 nM Fra a 1.02 antibody in T-buffer over the sensor and waiting until the binding signal went into saturation. Since Fra a 1 –DNA conjugates were not purified, there is always a mixture of unmodified and modified DNA on the surface. Dissociations of proteins were measured by rinsing buffer. His-tagged proteins Fra a 1.01E, Fra a 1.02, Fra a 1.03 and FaAP-EGFP were bound to NTA₃-tagged 48-x DNA in T-buffer. Full surface coverage was ensured by using a protein concentration of at least 200 nM throughout all experiments. For the protein sizing measurement²², time-resolved rising curves before and after saturation of NTA₃-DNA layers with His-tagged protein were used.

RESULTS

In silico analysis

As MdAP (*Mal d 1 Associated Protein*) has been described as interacting partner of two isoforms of the major apple allergen Mal d 1²¹, we screened the genome sequence of *F. vesca*¹⁶ for genes encoding homologous proteins. The *FaAP* gene (*Fra a 1 Associated Protein*) exhibits 69% protein sequence identity to *MdAP*²¹, is 579 bp long, and codes for a protein of 193 amino acids with a molecular weight of 21.461 kDa and a calculated pI of 4.49 [Fig. 2]. FaAP, in accordance with MdAP, is encoded by a single gene in the genome of the woodland strawberry.¹⁶ A BLAST search of the FaAP sequence at NCBI provided, amongst others, protein sequences from *Solanum lycopersicum* (79% identity; XM_004249348.1), *Prunus persica* (69%; EMJ03824.1), *Populus trichocarpa* (59%, XP_002323365.1), *Vitis*

vinifera (58%, XP_002264531.1) and *Arabidopsis thaliana* (46%, AAD21467.1). These protein sequences are described as hypothetical and predicted proteins, homologous to MdAP. The genomic DNA (gDNA) of *FaAP* consists of two exons that are separated by an intron. The intron starts after 204 bp with the conserved splice site GT, at codon 69, has a length of 1,282 bp and ends with the splice site AT. FaAP is presumably not secreted, is not a DNA binding protein and does not contain a nuclear localization signal as was predicted by Predict Protein³⁰ although it could enter the nucleus due to its small size.

Interaction between Fra a 1 proteins and FaAP via Y2H screen

To identify interaction partners of the Fra a 1 proteins, we performed Y2H analysis with Fra a 1.01E/1.02/1.03 and FaAP, as its ortholog from *M. × domestica* MdAP has been shown to bind to Mal d 1.²¹ The known interaction of Mal d 1 with MdAP and AMSH3 with VPS2.1 served as positive controls [Fig. S1].^{21,27} The combination of FaAP:Fra a 1.01E showed strong yeast cell growth whereas Fra a 1.03:FaAP primarily promoted cell growth when ‘bait’ and ‘prey’ vectors were interchanged. The combination empty GBD:FaAP and in particular the interchanged combination FaAP:empty GAD induced strong reporter gene auto-activity. Thus, a latent auto-activation activity was present in FaAP and could not be reduced using high concentrations of 3-AT (20 mM). The negative controls of Fra a 1.01E/1.02/1.03 in combination with empty GBD and empty GAD showed only a minor cell growth.

To reduce the auto-activity of FaAP, we divided FaAP in an N- (1-291 bp; FaAP-N) and C- (292-582 bp; FaAP-C) terminal part and tested interaction in different combinations. FaAP-N and the three Fra a 1 isoforms in both vector arrangements did not reconstitute a functional GAL4 transcription factor. However, when the combinations of FaAP-C with Fra a

1.01E/1.02/1.03 were analysed, FaAP-C:Fra a 1.01E was able to induce reporter gene expression.

The negative controls of FaAP-N and FaAP-C with the empty GBD and FaAP-N:empty GAD co-transformation did not support cell growth, but FaAP-C:empty GAD represented a strong transcriptional activator comparable with FaAP:empty GAD. This implies that the amino acid sequence which is responsible for the auto-activation activity of FaAP is primarily located in the C-terminal (292-582bp) fragment.

We also analysed Fra a 1:MdAP as well as Mal d 1:FaAP in different combinations to test whether FaAP and MdAP (69% amino acid identity with FaAP) share similar functions. Although reporter gene expression was observed for FaAP:Mal d 1 this result is probably owed to the auto-activation activity of FaAP.

Surprisingly, physical interaction of Mal d 1 and MdAP could not be unambiguously confirmed by the Y2H analysis but similar to FaAP, MdAP induced gene transcription in the presence of the empty GAD. However, auto-activation caused by MdAP seemed to be weaker compared to FaAP. The known association of AMSH3 (“prey”) and VPS2.1 (“bait”) served as positive control.²⁷ The results were reproduced and verified by β -galactosidase filter assays [Fig. S2].

Although the Y2H analysis provided some evidence for a physical interaction between Fra a 1 proteins and FaAP, the results could not clearly demonstrate the association due to a high auto-activation background of the C-terminal sequence of FaAP. Thus, an alternative and independent system was used for further analysis.

Interaction between Fra a 1 and FaAP by time-resolved DNA switching

A time-resolved DNA switching system was applied to analyse the putative binding of three Fra a 1 isoforms and FaAP.²² Double-stranded DNA (48-x) was immobilized on a

biochip and DNA orientations were measured in real-time by fluorescence energy transfer of the Cy3 dyes (covalently bound fluorescence dye) attached to the DNAs' distal end [Fig. 1]. Employing time-correlated single photon counting, the upward and downward motion of the DNA was monitored. A fluorescence histogram was acquired within seconds.

As a positive control, we first analysed the interaction of a polyclonal antibody raised against Fra a 1.02 with the three Fra a 1 isoforms bound to the 48-x DNA. The DNA lever motion slightly slowed down when the allergens were attached to the DNA, except for Fra a 1.01E [Fig 3; pink broken lines compared with the black lines]. The motion of the DNA lever decelerated even stronger in the presence of the antibody as shown by the decreasing slopes of the fluorescence response curves when the binding partner was added, indicating an interaction with the partner [Fig. 3; green broken lines]. Based on the different slopes it can be concluded that Fra a 1.02 showed the highest and most rapid interaction with the antibody whereas Fra a 1.03 exhibited a lower affinity to the polyclonal antibody. In the case of Fra a 1.01E the motion of the DNA lever decelerated in the presence of the antibody but the time-resolved rising curves for the unmodified DNA and the Fra a 1.01E-DNA conjugate were almost identical making the result inconclusive. The time courses can be fitted well with a single exponential function, which indicates reaction-limited association kinetics. The area under the curves is a parameter gauging the swiftness of the motion allowing the calculation of k_{on} (rate constant for association).²² As no dissociation was observed for Fra a 1.02 and Fra a 1.03 only the k_{on} was determined with $1.9 \pm 0.5 \cdot 10^5 \text{ M}^{-1} \text{ s}^{-1}$ and $0.6 \pm 0.2 \cdot 10^5 \text{ M}^{-1} \text{ s}^{-1}$, respectively [Table S1]. The k_{on} value for Fra a 1.01E was not calculated due to the inconclusive result.

When the Fra a 1.01E/1.02/1.03 proteins were covalently conjugated to the DNAs' top end the DNA lever motion slowed down as is visible by the smaller slopes of the curves for DNA-Fra a 1 (48x-Fra a 1.01E/1.02/1.03) fusions in comparison with the unconjugated

DNA (48-x) [Fig. 4]. To detect a possible interaction, of Fra a 1 proteins with FaAP, the Fra a 1 isoforms bound on the biochip were rinsed with 200 nM and 500 nM of FaAP-EGFP protein. When Fra a 1.01E, Fra a 1.02 and Fra a 1.03 were analysed in combination with FaAP-EGFP the movement of the DNA-protein-protein complex decelerated further [Fig. 4]. This indicates that the three Fra a 1 isoforms interact with FaAP-EGFP. EGFP did not bind to the Fra a proteins 1.01E and 1.02 as the fluorescence histograms in the presence and absence of EGFP were identical [Fig. S3]. The binding kinetics were calculated using the measured k_{on} and k_{off} values [Table 1]. Fra a 1.01E binds slow but very strong to FaAP-EGFP (K_D of 4.5 nM). The Fra a 1.02 and Fra a 1.03 binding to FaAP-EGFP exhibit nearly identical kinetics (K_D of 15 and 11 nM, respectively).

Determination of the hydrodynamic diameter of Fra a 1 and FaAP-EGFP by time-resolved DNA switching

In order to calculate the hydrodynamic diameter of the complexes, the three Fra a 1 isoforms and FaAP-EGFP were bound with their His-tag to NTA₃-DNA. Protein concentrations of 200 nM were used to saturate all NTA₃-chelator heads with His-tagged protein. FaAP-EGFP forms a complex with Fra a 1.01E, 1.02 and 1.03 as slopes of the fluorescence response curves were always the smallest in the presence of FaAP-EGFP [Fig. 5]. In the case of FaAP-EGFP and Fra a 1.01E, the switching dynamics of the DNA-protein-protein complex slowed down to a greater extent than in the interaction of FaAP-EGFP with the other Fra a 1 proteins, which indicates that Fra a 1.01E probably binds as a dimer [Fig. 5; green broken lines]. Similarly, Fra a 1.01E bound to NTA₃-DNA decelerated the motion of the DNA lever stronger than the two other Fra a 1 proteins which supports the hypothesis of the formation of Fra a 1.01E dimers. In the interaction study of Fra a 1.01E two time constants could be measured. A fast binding for Fra a 1.01E to the NTA₃-His-tag (~0.02

s⁻¹) and a slow but strong interaction between Fra a 1.01E and FaAP-EGFP (~ 0.003 s⁻¹), as described above.

To investigate the diverse binding characteristics, the hydrodynamic protein diameters of the NTA₃-DNA bound proteins were determined by the Lollipop model.²² Fra a 1.01E showed a visible larger shift of the fluorescence response curve [Fig. 6] and consequently a higher hydrodynamic protein diameter (5.5 nm) compared with Fra a 1.02 and Fra a 1.03 (4.6 and 4.2 nm), respectively [Table 2]. This is an indication that two Fra a 1.01E proteins are attached to the NTA₃-DNA and supports the hypothesis of a FaAP independent formation of a dimeric structure of Fra a 1.01E as already drawn from Figure 5(A). The hydrodynamic diameters of Fra a 1.02 and Fra a 1.03 was in accordance with their similar molecular masses. FaAP-EGFP showed also an appropriate hydrodynamic diameter for the molecular mass of the fusion protein [Table 2].²²

As a second positive control, in addition to the antibody association shown in Figure 3, the known protein-protein interaction between RCAR8 (UniProt # Q9FLB1), a Bet v 1 homologous protein, and ABI2 (GenBank Accession Number Y08965.1) in the presence of abscisic acid (ABA) was analysed.³¹ RCAR8 is a *Regulatory Component of ABA Receptor 8* and has been shown to interact in an ABA-dependent manner with ABI2 (*Abscisic acid Insensitive 2*), a PP2C (*protein phosphatase Type 2C*) phosphatase.^{31,32} After binding of ABI2 (50 nM) to NTA₃-DNA, a mixture of RCAR8 (100 nM) and ABA (10 μ M) was rinsed over the sensor. A clearly shifted time-resolved fluorescence response curve, indicative of an ABI2-ABA-RCAR8 complex, was observed [Fig. 4(D)]. The ABI2-ABA-RCAR8 complex exhibits a k_{on} of $3.2 \pm 0.8 \cdot 10^5$ M⁻¹ s⁻¹ and finally a K_D of 1.9 nM [Table 1]. After binding of ABI2 (50 nM) to NTA₃-DNA, RCAR8 (100 nM) was rinsed over the sensor and forms a complex with the immobilized ABI2 also in absence of ABA [Fig. 4(D)]. Thereafter a solution of ABI2 (50 nM), RCAR8 (100 nM) and ABA (10 μ M) was applied to the biochip,

but the time-resolved curve remained unchanged. This result indicated that the protein-protein interaction between ABI2 and RCAR8 is ABA-independent.

As negative controls we analyzed the binding of FaAP-EGFP, Fra a 1.01E, Fra a 1.02, Fra a 1.03, Fra a 1.02 antibody, RCAR8 and RCAR8 in the presence of ABI2 to unmodified 48-x DNA [Fig. 7]. An interaction was not observed, only FaAP-EGFP at a concentration of 500 nM and RCAR8-ABI2 exhibit a minor non-specific binding to the unmodified 48-x DNA.

DISCUSSION

In this study, FaAP was identified as an ortholog of MdAP by *in silico* analysis and the putative *in vivo* interaction between Fra a 1 and FaAP was analysed by an Y2H assay. However, strong auto-activation^{33,34} of FaAP, a well-known disadvantage of the Y2H system, was observed [Fig. 3]. Similarly, in this study, MdAP was identified as an activator of the reporter genes in the Y2H analysis in combination with an empty prey vector. Auto activation of MdAP has not been reported, yet.²¹ The weakness of the Y2H results called for additional ways to test the putative physical interaction of the Fra a 1 proteins and FaAP.

Thus, electro-switchable DNA biochips were chosen as an alternative method for the analysis of protein interaction by time-resolved measurements.²² The study provided a distinct indication for the physical association of Fra a 1.01E, Fra a 1.02 and Fra a 1.03 with FaAP, *in vitro*. The validity of the system was confirmed by determining the binding of the allergens to an allergen specific antibody and measuring the known physical interaction of RCAR8 and ABI2.³¹ The kinetic data indicated that Fra a 1.01E showed the strongest affinity to FaAP-EGFP followed by Fra a 1.03 and Fra a 1.02 [Table 1]. The interaction seemed to be isoform independent as FaAP interacts with three Fra a 1 isoforms.

Besides, Fra a 1.01E clearly showed a greater hydrodynamic diameter than Fra a 1.02 and Fra a 1.03 as was calculated by the Lollipop model [Fig. 6].²² Thus, we hypothesized, that two molecules of Fra a 1.01E bind to one DNA lever and Fra a 1.01E was detected as dimer. As Fra a 1.01E in complex with FaAP-EGFP displayed a much bigger shift compared to a minor shift of Fra a 1.02/1.03 in interaction with FaAP-EGFP [Fig. 5], we followed that a Fra a 1.01E dimer interacts with two FaAP-EGFP fusion proteins bound to two NTA₃-DNA levers. In this way the DNA strands interlink on the chip surface.²⁸

Recently, it was shown that Fra a 1.01E is a monomer in solution¹⁸ and in the crystal 3D structure.²⁰ In the present study, the time resolved DNA-switching measurement system demonstrated dimerization of Fra a 1.01E. Similarly, the most prominent representative of the PR-10 family, the major birch pollen allergen Bet v 1, isolated directly from birch pollen, forms a dimer.³⁵ The dimerization was confirmed by western blotting with a monoclonal antibody and both the monomer and dimer showed RNase activity. AmPR-10 from the Chinese medical plant *Astragalus mongholicus*¹ and SPE16 from *Pachyrrhizus erosus*, the Mexican yam⁵, form homodimers in solution and dimerization of a norcoclaurine synthase (NCS), another PR-10 allergen-related protein, seemed to be important for the enzyme activity.³⁶

The concentration of allergens and the oligomerization status of allergens have a tremendous effect on their allergenic potential. It was shown that the allergens are “inactive” in their low concentration status as monomers. At higher concentrations allergens are “active” as oligomers. Some important members of the PR-10 protein family like the birch pollen allergen Bet v 1, the apple allergen Mal d 1, the carrot allergen Dau c 1 and the celery allergen Api g1 exhibit a low allergenic potential as monomers. But at a higher oligomerization status, e.g. dimers, the allergenic potential increases and they can be identified as hyperallergenic proteins.³⁷ Lately, Fra a 1.01E was identified as a hypoallergenic

strawberry allergen in a basophil activation test as low concentrations (0.5 ng, 5 ng and 50 ng) were applied in the test.¹⁷ In addition, further BAT analyses were performed with a higher concentration of 500 ng recombinant Fra a 1 proteins. The increase of protein concentration led to an enormous rise of basophil activation with an enhancement of 69% between 50 ng and 500 ng recombinant protein Fra a 1.01E applied in the test [Fig. S4]. In contrast, Fra a 1.02 and Fra a 1.03 exhibited only a minor increase in basophil activation between 50 ng and 500 ng. Hence, we assume that dimerization as shown by the time resolved DNA-switching measurement and the significantly increased allergenic potential of Fra a 1.01E at high concentration (500 ng) is accompanied by a significant increase of the allergenic potential. It seems that the oligomerization status of the strawberry allergen significantly affects the allergenic potential of the protein.

Another outstanding example for PR-10 proteins and homologues of Bet v 1 are the RCAR proteins, comprising 14 members. Four RCARs (RCAR11, 12, 13 and 14) form dimeric structures in solution.³⁸⁻⁴¹ The RCAR proteins contain the conserved loop 2 (CL2) which undergoes, after ABA binding, a conformational rearrangement for creating the interaction surface for PP2C. In the dimeric structure of RCAR14 the switching CL2 may be constrained by the dimer. Consequently the dimeric structure prevents the binding of ABA and the interaction of PP2C with RCAR14.⁴⁰ So far, the functional importance of the dimerization of PR-10 proteins could not be established.

In the case of Fra a 1.01E the physical interaction of the dimer with FaAP could have an important physiological impact similar to the enzymatic activity of the NCS dimer³⁶ and the inhibition of the RCAR14-ABA-PP2C interaction by the RCAR14 dimer, which affects the downstream ABA-pathway.⁴⁰ As PR-10 related proteins form a cavity for the binding of ligands such as phytosteroids and flavonoids^{42,43} it is conceivable that they act as plant hormone and secondary metabolite carriers. Multimeric complexes involving orthologous of

FaAP might have an essential function in trafficking the metabolites. Similarly, the cytosolic complex of a PR-10 protein and a leucine-rich repeat protein 1 (LRR1) from pepper (*Capsicum annuum*) has been shown to be responsible for cell death-mediated defense signalling.⁴⁴ LRR1 acts as positive regulator of hypersensitive cell death triggered by PR-10.

The determination of the binding between Fra a 1.01E/1.02/1.03 and the polyclonal Fra a 1.02 antibody served as a reliable positive control and test for the specificity of the antibody. The results of the time resolved DNA-switching measurements reflect very well the amino acid sequence identities of the protein sequence. Fra a 1.02 shows the highest and most rapid interaction with the antibody, followed by Fra a 1.03 (80.1% identity) and Fra a 1.01E (78.9% identity). The known association of RCAR8-ABA-ABI2 was used as second positive control. The strong binding between these three components was already reported.³¹ The interaction of the complex in the presence of ABA could be verified (K_D of 1.9 nM). However, even without the presence of ABA, RCAR8 bound to ABI2. This result indicates that RCAR8 and ABI2 can interact also in an ABA-independent manner.

The protein FaAP has an orthologue protein in the model plant *Arabidopsis thaliana* with a protein sequence identity of 46%. The orthologue protein AtAP (AAD21467.1; Locus detail at TAIR: AT2G35900) is temporarily described as a hypothetical protein. In an extensive study to investigate the network evolution of the *Arabidopsis* interactome the hypothetical protein AtAP was involved in a Two-hybrid interaction study. AtAP as the prey (transcriptional activation domain) partner stroked up an interaction with two proteins Atg8d and Atg8f which represented the bait (DNA binding domain) part in the Two-hybrid system. The proteins Atg8d/f (Locus detail at TAIR: AT4G16520, AT2G05630) are described as autophagy-related proteins 8d/f.⁴⁵⁻⁴⁸ The Atg8 protein family which encodes ubiquitin-like proteins participate in various stages of the important autophagic process like intracellular membrane trafficking.⁴⁹ Only recently it was reported that a trafficking pathway of

anthocyanins, co-localized in an endomembrane system, overlaps with the endoplasmic reticulum-to-vacuole protein sorting way in *Arabidopsis* and concurs to the formation of vacuolar inclusions. This is a novel and interesting mechanism of vesicular transport and vacuolar sequestration of anthocyanins.⁵⁰

It was shown that the *Fra a 1* expression is directly linked to flavonoid biosynthesis and that the *Fra a 1* allergen has an essential biological function in pigment formation in strawberry fruits.¹⁵ This was demonstrated by transient RNAi-mediated silencing experiments^{19,51}, structural analysis studies^{20,52} and proteome analysis of a naturally white-fruited strawberry variety¹⁴. The interaction between the *Fra a 1* isoforms and FaAP and the association between *Arabidopsis* AtAP (FaAP orthologue protein) and Atg8d/f provide a first clue for the biological function of *Fra a 1*. The presumed association of the three proteins (*Fra a 1*, FaAP and Atg8d/f) and the *Fra a 1* dependent pigment formation may be related to the transformation of vacuoles during fruit development, which serve as storage compartments for anthocyanin pigments.

CONCLUSIONS

FaAP could be confirmed as a putative interaction partner of the strawberry allergen *Fra a 1.01E/1.02/1.03*. Although an Y2H assay provided first evidence of a physical interaction, strong auto-activation activity of the C-terminal domain of FaAP rendered the results inconclusive. However, measurements by a DNA switching device provided evidence for the protein-protein binding of FaAP and *Fra a 1* isomers as well as RCAR8, ABI2 and abscisic acid. The data provide a foundation for further research on the physiological function of PR-10 proteins in developmental processes, secondary metabolism and antimicrobial activity.

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Abbreviations: ABI2, *Abscisic Acid Insensitive 2*; ABA, *abscisic acid*; FaAP-EGFP, *Fra a* 1-Associated Protein-Enhanced Green Fluorescent Protein; *Fra a* 1.01E/1.02/1.03, *Fragaria x ananassa* 1.01E/1.02/1.03; GDB, *GAL4 binding domain*; GAD, *GAL4 activation domain*; ITO, indium-tin-oxide; RCAR8 (PYL5), *Regulatory Components of ABA Receptor 8* (*Pyrabactin Resistance 1-Like 5*); 3-AT, 3-amino-1,2,4-triazole (Sigma-Aldrich).

This article contains supplemental Tables S1 and Figures S1 to S4.

TABLE and FIGURE LEGENDS

Table 1: Kinetic data obtained by the time-resolved DNA switching system for the interaction between FaAP-EGFP and the three *Fra a* 1 isoforms and ABI2, RCAR8 and ABA.

Table 2 Hydrodynamic diameter of Fra a 1.01E/1.02/1.03 and FaAP-EGFP measured via the lollipop model.

Figure 1 Schematic presentation of a DNA lever with the protein Fra a 1.03 (D_H : effective protein diameter) bound by the capture molecule, NTA₃ (green), which is covalently attached to one single strand. The second single strand comprises the fluorescence molecule (yellow star). The dotted line represents the “lollipop” silhouette for calculating the hydrodynamic diameter (D_H).

Figure 2 Protein sequence alignment³⁰ of orthologous proteins to MdAP in the strawberry genome FaAP (69%, KF785812) and further orthologous (hypothetical and uncharacterized) proteins in *Vitis vinifera* (58%, XP_002264531.1), *Arabidopsis thaliana* (46%, AAD21467.1), *Populus trichocarpa* (59%, XP_002323365.1), *Solanum lycopersicum* (79% identity; XM_004249348.1) and *Prunus persica* (69%; EMJ03824.1). Legend of symbols: accordance of nucleotides (.); absence of nucleotides (-); large sequence logo letters indicate the same nucleotide in all compared sequences; small/smaller sequence logo letters indicate different nucleotides in the compared sequences.

Figure 3 Time-resolved rising curves for unmodified DNA in buffer (48-x, black line); A-C: Fra a 1.01E/1.02/1.03-DNA conjugates (purple dashed line), Fra a 1.01E/1.02/1.03-DNA conjugates after rinsing a solution of 50 nM polyclonal Fra a 1.02 antibody over the sensor and the formation of a complex (green dotted line). Axis labelling: Y-axis: normalized fluorescence; X-axis: time in μ s.

Figure 4 Time-resolved rising curves for unmodified DNA (48-x, black line); A-C: 48x-Fra a 1.01E/1.02/1.03 conjugates (purple dashed line), 48x-Fra a 1.01E/1.02/1.03 conjugates after rinsing a solution of 200 nM FaAP-EGFP over the sensor and the formation of a complex (green dotted line); D: ABI2-NTA₃-modified DNA conjugate (50 nM; purple dashed line),

RCAR8 (100 nM) forms a complex with the immobilized ABI2 also in absence of ABA (blue dashed dotted line); flowing a solution of ABI2 (50 nM), RCAR8 (100 nM) and ABA (10 μ M) over the NTA₃-modified DNA levers on the sensor leads to no further shift in the time-resolved curve (green dotted line). Axis labelling: Y-axis: normalized fluorescence; X-axis: time in μ s.

Figure 5 Time-resolved rising curves for NTA₃-DNA after Ni-loading in buffer (black line); A-C: Fra a 1.01E/1.02/1.03-NTA₃-DNA conjugates (purple dashed line), FaAP-EGFP-NTA₃-DNA conjugates (blue dashed dotted line), mixture of Fra a 1.01E/1.02/1.03 and FaAP-EGFP-NTA₃-DNA conjugates (green dotted line); protein concentrations were 200 nM for saturating all the NTA₃-chelator heads with His-tagged protein; Fra a 1.01E in complex with FaAP-EGFP display a bigger shift compared to a minor shift of Fra a 1.02/1.03 in interaction with FaAP-EGFP; the comparisons of the diverse shift characteristic leads to the indication of a possible dimer formation of Fra a 1.01E. Axis labelling: Y-axis: normalized fluorescence; X-axis: time in μ s.

Figure 6 Time-resolved rising curves for NTA₃-DNA after Ni-loading in buffer (black line); A-C: Fra a 1.01E/1.02/1.03-NTA₃-DNA conjugates and (D) FaAP-EGFP-NTA₃-DNA conjugate (purple dashed line); (A) Fra a 1.01E and (D) FaAP-EGFP bound to NTA₃-DNA show a bigger shift than (B,C) Fra a 1.02/1.03; the bigger shift of Fra a 1.01E give rise to the assumption that there is an interlinking on the NTA₃-DNA chip surface through a Fra a 1.01E dimer; Fra a 1.02/1.03 have a similar size; FaAP-EGFP displays a bigger shift based on the higher molecular size and the two available His-tags. Axis labelling: Y-axis: normalized fluorescence; X-axis: time in μ s.

Figure 7 Time-resolved rising curves for unmodified DNA in buffer (48-x, black line). No interaction between respectively (A,B) Fra a 1.01E/1.02 (200 nM; purple dashed line), (B)

Fra a 1.03 (200 nM; green dotted line), (C) polyclonal Fra a 1.02 antibody (50 nM; purple dashed line), (C) FaAP-EGFP (200 nM; green dotted line) and unmodified 48-x DNA; (D) Minor non-specific binding of FaAP-EGFP (500 nM; purple dashed line) to unmodified 48-x DNA; (E) No interaction between RCAR8 (100 nM; purple dashed line) and unmodified 48-x DNA; (E) Minor non-specific interaction of ABI2-RCAR8 (50 nM/100 nM; green dotted line) to unmodified 48-x DNA. Axis labelling: Y-axis: normalized fluorescence; X-axis: time in μ s.

Table 1: Kinetic data obtained by the time-resolved DNA switching system for the interaction between FaAP-EGFP and the three Fra a 1 isoforms and ABI2, RCAR8 and ABA.

	$k_{on} (M^{-1} s^{-1})$	$k_{off} (s^{-1})$	$K_D (nM)$
FaAP-EGFP – Fra a 1.01E	$5.8 * 10^3 \pm 0.4$	$2.6 * 10^{-5} \pm 0.6$	4.5 ± 1.1
FaAP-EGFP – Fra a 1.02	$1.2 * 10^5 \pm 0.1$	0.0017 ± 0.0002	15 ± 3
FaAP-EGFP – Fra a 1.03	$1.2 * 10^5 \pm 0.1$	0.0013 ± 0.0002	11 ± 2
ABI2 – RCAR8 – ABA	$3.2 * 10^5 \pm 0.8$	$6.0 * 10^{-4} \pm 1.0$	1.9 ± 0.5

Table 2 Hydrodynamic diameter of Fra a 1.01E/1.02/1.03 and FaAP-EGFP measured via the lollipop model.

	mass (g mol ⁻¹)	hydrodynamic diameter (nm)
Fra a 1.01E	18696	5.5 ± 0.5
Fra a 1.02	18428	4.6 ± 0.2
Fra a 1.03	18378	4.2 ± 0.2
FaAP-EGFP	48384	6.7 ± 0.3





