

Hypoallergenic profilin: a new way to identify allergenic determinants

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ABSTRACT

Plant profilins are important panallergens, contributing to numerous food and pollen allergies. They fulfil essential functions in all eukaryotic cells, including yeast and mammals, making breeding of profilin-deficient crop plants an impossible task. To obtain hypoallergenic profilin-variants a novel yeast based screening system to select for fully active but hypoallergenic profilins was developed. Despite similar biological functions plant profilins share only limited amino acid sequence identity to non-plant profilins. Thus it should be possible to select for hypoallergenic profilin variants which have kept their biological function. These variants were subsequently tested for IgE-binding and their 3D folding was analysed. As prerequisites we developed a conditional profilin-deficient yeast mutant strain and made use of a newly discovered profilin variant from tomato which enabled pre-selection for efficient mutagenesis. Following random mutagenesis and transformation of tomato profilin (Lyc e 1) into the designed yeast strain, we were able to screen for functional tomato profilin variants. Testing these variants for IgE binding revealed that two variants showed a strong reduction in IgE binding of four Lyc e 1 sensitized patients and one birch pollen allergic patient. This result, for the first time, provides strong evidence that selection of hypoallergenic Lyc e 1 variants is possible. Furthermore, this knowledge provides the basis for molecular breeding of hypoallergenic profilin alleles in tomato.

Keywords: Lyc e 1, PFY1, tomato allergy, birch allergy, profilin

ABBREVIATIONS

ECL: enhanced chemiluminescence

5-FOA: 5-Fluoroorotic acid monohydrate

His: histidine

LiAc: lithium acetate

Leu: leucine

Lyc e 1: tomato profilin

mRNA: messenger RNA

OAS: Oral Allergy Syndrome

PFY1: yeast profilin

RNAi: RNA interference

S. cerevisiae: *Saccharomyces cerevisiae*

TALEN: Transcription activator-like effectors nucleases

TBS: Tris-buffered saline

TILLING: Targeting Induced Local Lesions in Genomes

Ura: uracil

WT: wild type

INTRODUCTION

Food allergy imposes a significant health, emotional, and economic burden on people affected and their families [1]. Individuals with food allergy are at risk of anaphylactic reactions if they ingest food to which they are sensitive. Reactions of food allergy range from mild symptoms as Oral Allergy Syndrome (OAS) to life-threatening anaphylactic shock [2].

Among the most important allergen families the profilins range on third place (ALLFam; Stand: Dec. 2011). Profilins are 12-15 kDa monomeric proteins found in all eukaryotic cells, from human to yeast. They act as actin- and phosphatidylinositol 4,5-bisphosphate-binding molecules involved in the regulation of the cytoskeleton and signal transduction. Their allergenic nature was discovered by the ability of Bet v 2 to bind IgE from birch pollen allergic individuals [3]. Meanwhile profilins are recognized as panallergens, playing an important role as latex, pollen and food allergens [4]. In tomato, profilin is identified as minor allergen in fruits, showing IgE reactivity in 22% to 26% of patients with tomato allergy [5], [6]. Up to date, three isoforms were described, the first one was LycPro1 which is mainly expressed in pollen [7] and two other isoforms with allergenic properties (Lyc e 1.01 and Lyc e 1.02) were found in different tissues including fruits [5], [6].

Since a specific immunotherapy for food allergy is not available [8], different methods to produce hypoallergenic food were persecuted, involving enzymatic or physical processing of allergic proteins [9] and gene silencing including RNA interference (RNAi). RNAi has been successfully used to decrease the accumulation of allergenic proteins in soybean, rice, apple, tomato, peanut [10] and carrot [11]. In previous studies [12], [13], [14] we successfully silenced Lyc e 1 (profilin) and Lyc e 3 (nonspecific lipid transfer protein) in transgenic tomato plants. While growth of Lyc e 3-silenced tomato plants was not affected [12] the Lyc e 1-silenced plants were characterized by severe growth retardations, delayed flowering and less fruit formation, due to the abrogation of the essential cellular function of profilin [13]. Apart from the moderately low similarity in amino acid sequences (22%-44%) plant and non-plant profilins have functional similarities. Plant profilins could interact with mammalian actins *in vitro* [15], [16] while the complementation of a yeast profilin-deficient mutant by plant profilins was first shown by expression of *Arabidopsis thaliana* profilin PFN1 and PFN3 and maize profilin in Christensen *et al.*, [17]. Despite the sequence divergence between the profilins of yeast and *Arabidopsis*, the *Arabidopsis* profilins could interact with actin and

substitute for maintaining cell division and growth. Meanwhile, we could restore the cellular function of profilin in transgenic Lyc e 1 tomato plants by simultaneous expression of yeast profilin (PFY1), which does not possess any identified allergenic properties, demonstrating the feasibility of creating low-allergenic food even if the allergen fulfils essential cellular functions. The co-expression of PFY1 restored growth and biomass production almost to wild type levels [18].

Another possibility for the reduction of allergenic proteins is the modification of IgE epitopes of allergens. IgE antibodies recognize specific surface structures of allergens. If these structures are successfully changed, they cannot be recognized by allergen specific IgE antibodies and hence no allergenic reaction will be triggered. Identification and modification of IgE epitopes was reported for Bet v 2 [19] and its homologues in sunflower [20], latex [21], melon [22], [23], [24] and peanut [25], functionality of the mutated proteins, however, was not considered in these studies. In order to combine both, functionality and hypoallergenicity, we developed a novel yeast-based screening system for profilin mutants. The system allowed for the enrichment of mutated but functional Lyc e 1-variants. Subsequently, these variants were tested for IgE-binding using antisera from Lyc e 1 allergic patients. Using this system we could identify two Lyc e 1-variants showing a strong reduction of IgE binding capacity in at least three patients, but having full biological activity.

RESULTS

Design of a yeast-based system to select for functional Lyc e 1 variants

Establishment of a cell-based selection system for hypoallergenic but functional profilin variants is non-trivial since a diminished profilin expression was shown to have drastic effects on growth and viability in different organisms [13] [26] and requires two prerequisites: (i) a suitable profilin-deficient mutant and (ii) a pre-selection screen for mutations in the target gene. The outline of our strategy is depicted in **Figure 1**.

Establishing profilin-deficient yeast strains for complementation

First attempts trying to establish a profilin knock-out yeast strain, suitable as selection system, failed. Considering the essential function of profilin in eukaryotic cells, this observation may not be that surprising. To nevertheless establish a genomic yeast profilin deletion mutant (Δ pfy1) we followed a two-step procedure. First, wild type yeast BY4741 was transformed with pYES2 carrying the PFY1 coding region under control of the galactose promoter. To study whether expression of PFY1 in yeast was successful, yeast colonies transformed with pYES2-PFY1 were analysed by immunoblotting using a cross-reactive anti-profilin antibody. The molecular weight of PFY1 is denoted with 13,677 kDa, the recombinant PFY1 band migrated at about 14 kDa. The GAL1-promoter was induced by the transfer of the yeast cells to galactose containing medium and expression was visualized over a time period of 24 hours (**Supplemental Figure 1**). Expression of PFY1 in BY4741/pYES2-PFY1 yeast cells could be detected by an increase in the PFY1 protein abundance, starting four hours after induction.

Subsequently, the chromosomal PFY1 gene was replaced by the His3 marker gene and selection of Δ pfy1 mutants was carried out on uracil and histidine deficient medium. Only Δ pfy1 mutants carrying the pYES2-PFY1 were able to grow on selection medium (**Supplemental Figure 2A**). To verify the replacement of the chromosomal PFY1, the BY4741/pYES2-PFY1/ Δ pfy1 yeast cells were tested via genomic PCR using oligonucleotides binding up- and downstream of the recombination site. The results of the PCR were compared with BY4741 wild type yeast PCR results. The PCR showed that using BY4741/pYES2-PFY1/ Δ pfy1 as template gives three amplification products in the range of 1026 bp, 732 bp and 410 bp, whereas using BY4741 as template, only one amplification product of 1029 bp

could be detected (**Supplemental Figure 2B**). Using the bypass strategy described, a chromosomal Δ pfy1 mutant could be established.

Random mutagenesis and selection of functional Lyc e 1.02 variants

Since the immunological validation of potential profilin mutants requires protein isolation and testing from single yeast colonies, a pre-selection step for mutagenesis is required. Assuming, that a large number of complementing cDNAs simply escaped mutagenesis, identification of mutants is jeopardized. To achieve this prerequisite we made use of natural variation of profilin sequences. Comparing profilin sequences from a wide range of sources reveals a conserved amino acid motive “VWA” at amino acid position 29-31 (position is based on yeast profilin). Interestingly, all *Solanaceae* species tested so far contain profilin isoforms which deviate from this conserved motive. These *Solanaceae* isoforms contain an arginine (R) instead of the conserved tryptophan (W) residue. Modelling the tertiary structure of both profilin variants using swiss pdb-viewer showed slight differences in protein tertiary structure possibly leading to alterations in protein function. Attempts to use tomato profilin Lyc e 1.02, carrying the tryptophan to arginine exchange, for complementation of yeast profilin mutant, failed, suggesting that the VWA motif is essential for profilin function in yeast. To validate this hypothesis, Lyc e 1.02R33 was mutagenized to Lyc e 1.02W33. Subsequently, Lyc e 1.02W33 was used to complement a profilin deficient yeast strain. Analysing the transformed yeast cells revealed that expression of Lyc e 1.02W33 restored normal yeast growth. These results are in agreement with an essential role of the conserved tryptophan residue in yeast. Furthermore, the data show that Lyc e 1.02 only complements profilin-deficient yeast cells following conversion of the arginine to tryptophan. Therefore, using Lyc e 1.02 in mutant screens allowed for the positive selection of mutated Lyc e 1.02 variants. To this end Lyc e 1.02 was subjected to *in vitro* random mutagenesis. The vector p425-GPD with the GPD

(glyceraldehyde-3-phosphate-dehydrogenase) promoter and the Leu2 gene as selection marker is a expression vector, which can be used for controlled expression of heterologous proteins in yeast [27].

For expression of Lyc e 1.02 variants under control of the yeast profilin promoter, the PFN promoter was cloned into the p425-vector to yield the p425P_{PFY} plasmid. Subsequently, the Lyc e 1.02 gene was cloned in the p425P_{PFY} plasmid which resulted in the construct p425P_{PFY}Lyc e 1.02. The p425P_{PFY}Lyc e 1.02R33 was used as a template for random mutagenesis (see materials and methods) of Lyc e 1.02, to avoid the complementation of non-mutated Lyc e 1.02 variants. The mutated Lyc e 1.02 PCR products were co-transformed with linearized p425P_{PFY} in the yeast strain BY4741/pYES2-PFY1/ Δ pfy1 after the Gap repair method [28]. The homologous recombination leads to the ligation of the linearized plasmid with the Lyc e 1.02 variants. The homologous regions were located on the 5'- and the 3'- end leading to the insertion of the Lyc e 1.02 fragment in the vector. As control BY4741/pYES2-PFY1/ Δ pfy1 yeast cells were transformed with p425P_{PFY}Lyc e 1.02 W33.

The Gap repair method offers the opportunity to test a huge amount of different Lyc e 1.02 variants at the same time in yeast for their ability to complement the profilin deficiency. Transformed cells were selected on his and leu deficient medium. To select for the loss of the extra-chromosomal PFY1 gene, transformed cells were transferred to medium containing 5-FOA, which was used to counter select the pYES2-PFY1 plasmid expressing the ura3 gene. Under these conditions only cells containing biologically functional Lyc e 1.02 variants, which can complement the pfy1 knock-out, but lacking the pYES2 vector can grow. **Supplementary Figure 3** shows the growth on SCA-medium –his-leu with and without 5'FOA. This selection revealed approx. 60 growing yeast colonies.

Sequence analysis of Lyc e 1.02 variants and *in silico* protein modelling

The obtained mutagenized Lyc e 1.02 fragments were subjected to sequence analysis. We could identify 40 mutants in which the mutations lead to amino acid changes in the sequence.

Figure 2 shows the results of 40 Lyc e 1.02 variants after error-prone PCR and sequencing.

As expected, all variants had the essential amino acid exchange, R33 to W33. Variant Lyc e 1.02-5 carried two additional amino acid exchanges, isoleucine¹⁵ to threonine and histidine¹⁹ to arginine. In mutant Lyc e 1.02-13 a C to T transition at position 142 lead to a threonine⁴⁸ to serine conversion. The mutant Lyc e 1.02-17 harbors two mutations, asparagine³⁸ to serine³⁸ and isoleucine¹¹⁸ to phenylalanine. We found several other mutants with silent mutations, which were not included in **Figure 2**. To get an idea of possible structural changes, Lyc e 1 wild type (**Supp. Fig. 4A, C, E**) and mutant protein models (**Supp. Fig. 4B, D, F**) were *in silico* generated on the basis of profilin I from *Arabidopsis thaliana* (PDB code 3NUL). They exhibit the typical profilin fold [29] which consists of a central 7-stranded β sheet (β 1, residues 22-27; β 2, 32-35; β 3, 65-67; β 4, 70-78; β 5, 82-87; β 6, 90-96; β 7, 100-106) sandwiched between the N- and C-terminal helices α 1 (residues 3-10) and α 3 (112-129) on one side and another helix α 2 (residues 44-55) on the other side. A structural alignment with profilin from *Saccharomyces cerevisiae* (PFY1, PDB code 1YPR, 33% sequence identity) using the DaliLite program [30] resulted in a root-mean-square deviation of 1.4 Å (for C α atoms) indicating a high degree of structural similarity. The largest difference between the Lyc e 1 and PFY1 profilins could be found in the loop region between helix α 1 and β strand β 1. In the PFY1 structure this loop is shortened by 4 residues. Interestingly, two of the Lyc e 1 mutations identified, namely I15T and H19R are also located in this loop region (**Fig. 3:**

Lyc e 1.02-5 depicted in yellow, **Supp. Fig. 4D**). While His19 is highly solvent accessible, Ile15 is only partly solvent accessible and in van der Waals contact distance to Ile118, a further mutation (I118F) identified (**Fig. 3: Lyc e 1.02-17** depicted in green, **Supp. Fig. 4F**). Ile118 is completely buried within the protein core. The remaining mutation, N38S is located in the loop region between β strand $\beta 2$ and helix $\alpha 2$, being again highly solvent accessible (**Fig. 3: Lyc e 1.02-17** depicted in green, **Supp. Fig. 4F**). The additional mutation in the mutant **Lyc e 1.02-13**, T48S can be found in helix $\alpha 2$, and is predicted to be solvent accessible (**Fig. 3: Lyc e 1.02-13** depicted in blue, **Supp. Fig. 4B**).

Lyc e 1.02 variants exhibit a reduced allergenic potential as shown by *in vitro* IgE immunoblotting

Different symptomatic tomato allergic patients and one birch pollen allergic patient with profilin-specific IgE antibodies were recruited to test for the allergenic potential of **Lyc e 1.02** expressing yeasts. **Table 1** shows their demographic data, allergic symptoms and the patient history as well as specific IgE levels. For IgE immunoblot analysis, protein extracts of yeast wild type as well as **Lyc e 1.02** expressing yeasts were separated on SDS-PAGE and blotted onto nitrocellulose membranes. The membranes were first incubated in diluted patient's serum, and then transferred into a diluted anti-human IgE antibody, conjugated with peroxidase. Sera from strong profilin-reactive tomato allergic patients T 002 (**Fig. 4A**) and T 045 (**Fig. 4B**) and weak profilin-reactive tomato allergic patients T 046 (**Fig. 4C**) and T 081 (**Fig. 4D**) showed no IgE binding to proteins from wild type yeast protein extracts (lane 1). However, both sera positively reacted with un-mutated **Lyc e 1.02** in protein extracts from transformed yeast cells (**Fig. 4**, lane 2). This binding was strongly reduced in extracts from transformed yeast cells carrying **Lyc e 1.02-5** (**Fig. 4**, lane 3) and **Lyc e 1.02-17** (**Fig. 4**, lane 5), whereas no reduction of the IgE binding capacity could be observed with extracts of **Lyc e**

1.02-13 expressing yeast cells (**Fig. 4**, lane 4). Similarly, serum IgE from profilin-reactive birch pollen allergic patient B 009, showed specific binding to un-mutated Lyc e 1.02 (**Fig. 4E**, lane 2), Lyc e 1.02-13 and recombinant Lyc e 1 (lane 6), but not to Lyc e 1.02-5 and Lyc e 1.02-17. To exclude that the different profilin variants accumulate to variable degree in yeast cells, IgG immunoblotting was carried out. As shown in **Figure 4F**, all profilin variants accumulated to similar amounts.

DISCUSSION

Currently, avoidance of the allergenic food is the gold standard for treatment of food allergy, although it often is difficult to manage and may cause impairment of quality of life, social activities and even mal nutrition [1].

As alternatives for these strict avoidance measures several approaches have been investigated: The RNAi technology was found to be a stable and reliable tool for the reduction of allergenic proteins in different plants [10], but silencing of essential genes has its limitations. For instance, silencing of profilin (Lyc e 1) in transgenic tomato plants led to smaller fruits and a dwarf phenotype [13]. To compensate for the loss of profilin in Lyc e 1 silenced plants, profilin-deficient transgenic tomato plants were complemented with yeast profilin. Analysis of the resulting transgenic plants revealed an almost complete restoration of plant growth and biomass production [18]. Although these experiments demonstrate the feasibility of generating hypoallergenic food even if essential proteins are targeted, consumer acceptance is likely to be low, since there are concerns that introducing foreign genes into crop plants may have an unexpected and negative impact on human health. To circumvent these fears, identification and modification of relevant IgE-binding epitopes and expression of functional but hypoallergenic profilin from the same species would be ideal.

To obtain hypoallergenic and profilin variants that fulfil all their cellular functions we used a novel yeast-based screening system. Previously it was shown that Arabidopsis profilins PFN1 and PFN3 as well as maize profilin were able to complement the growth defect of a yeast mutant with decreased profilin accumulation [17]. Owing to the fact that the homology between Arabidopsis and tomato profilin is about 75%, a complementation of profilin deficient yeast should be practicable with Lyc e 1 as well.

First attempts to generate a conventional PFY1 yeast knock-out strain were not successful. This is most likely due to the essential cellular function of profilin in eukaryotic cells. Support for this hypothesis comes from experiments with profilin knock-out mice, showing that homozygous profilin deficient embryos die in the 4-cell stage [26]. To create a non-lethal chromosomal profilin yeast deletion strain, wild type yeast was transformed with a plasmid providing an extra-chromosomal PFY1 gene. This transformed yeast strain was subsequently used for disruption of the PFY1 gene.

Random mutagenesis of tomato profilin Lyc e 1.02

Several B epitopes of profilins, both sequential and conformational, have been described using different approaches. The actin-binding site and the adjacent plant-specific pocket were found as an immunogenic region in many cases. Two regions overlapping with the actin-binding site were identified as major cross-reactive epitopes, and a third site consisting of residues 30-50 was found to be involved in the cross-reactivity in birch pollen profilin [19]. Three epitopes at the C-terminal helix (residues 100-10, 108-115 and 122-127) overlapping with the actin-binding site, and another epitope formed by segments 40-50 were found in sunflower profilin [20]. Several epitopes, which in fact cover most of the surface, have been identified in model structures of several profilins. Radauer *et al.* [21] highlighted three main candidates, whereas Leitner *et al.* [31] found that a circular peptide inhibited IgE binding to

mugwort pollen, birch pollen and celery tuber profilin and speculated that this mimotope might be an important epitope in plant profilins.

The characterization of IgE epitopes from the profilin Cuc m 2, the major melon allergen, has been addressed by means of two complementary strategies. Using overlapping synthetic peptides bound to a membrane, four major epitopes were identified: two with stronger IgE-binding capacity (E1: 66-75 and 81-93, and E2: 95-99 and 122-131) partially overlapping with the actin-binding site, and two weak epitopes (E3: 2-10 and E4: 35-45) [22]. In addition, an IgE conformational epitope was defined [23] which was highly conserved in birch Bet v 2 and timothy grass pollen Phl p 12 profilins. Cellular functionality of the resulting profilin variants was never tested in the experiments described above.

Taken together these studies imply that IgE-binding sites are scattered across the entire profilin protein making the design of functional and hypoallergenic profilin variants a difficult enterprise. Nevertheless, using tomato profilin as model we attempted to select for functional profilin with reduced affinity to specific IgE from tomato allergic patients. To this end yeast mutants were complemented with mutagenized tomato profilin. In this study the error-prone PCR was used for mutagenesis [32]. After cloning of mutated PCR products into the expression vector, the resulting mutant library was screened for changes in protein sequence of Lyc e 1.02. Using this kind of random mutagenesis, eight possible transversions, which means a change from purine to pyrimidine and vice versa, and four possible transitions, leading to a purine to purine or pyrimidine to pyrimidine change, are likely to happen. The analysis of the sequence revealed that different mutations in Lyc e 1.02 had been produced, whereas some of them were more prominent than others. All 40 tested Lyc e 1.02 variants displayed the one essential amino acid change for complementation, from R33 to W33. Additionally we could identify 17 mutants which had a switch in their amino acid sequence from N38 to S38 as well as I118 to F118 (Lyc e 1.02-17), 4 mutants with an amino acid change from T48 to S48 (Lyc e 1.02-13) and 2 mutants with the 2 amino acid changes from

I15 to T15 and H19 to R19 (Lyc e 1.02-5). These different mutations in the transgenic yeast strains resulted from three different transitions and only one transversion. Mutations in the remaining 17 variants did not result in amino acid changes.

Screening for IgE-binding revealed no different IgE-reactivity of the Lyc e 1.02 variants carrying silent mutations and mutant Lyc e 1.02-13. The remaining two mutants showed reduced binding capacity to sera from four tomato and one birch pollen allergic patient. This implies similar binding-sites at least for the IgEs tested and might explain cross reactivity of sera from patients sensitized to tomato and birch pollen profilin. Future experiments will be required to test more sera from patients sensitized to profilin from different sources and to select for tomato mutants carrying hypoallergenic profilin variants by TILLING (Targeting Induced Local Lesions in Genomes) or the application of TALENs (Transcription activator-like effectors nucleases). TILLING combines an EMS- (ethyl methanesulfonate) mutagenesis with DNA screening methods that identify point mutations in target genes. After successful identification of candidates, out-crossing provides the opportunity to get rid of unintended mutations. In a recent proof-of-concept study, TILLING was used to reduce allergen content in peanut [33]. TALENs are tools for generating site-specific double strand breaks, even in plant cells. Thus, TALENs can be used to achieve site-specific gene deletion or inactivation, and might be a new powerful agricultural biotechnology tool for crop improvement in the future [34].

MATERIAL AND METHODS

Selection and characterization of patients with tomato allergy

Approval of the local ethics committees (Faculty of Medicine, University of Erlangen-Nuremberg, No. 3344) for this study and written informed consent of all participants were obtained.

Sera were obtained from German patients with known type I-sensitization to profilin and allergic symptoms upon the ingestion of tomato fruits (n = 4), and a profilin sensitive birch pollen allergic patient. The diagnosis of tomato allergy was based on a positive case history, positive skin prick test and detection of specific IgE to tomato extract *in vitro*. Tomato specific IgE was determined by the CAP FEIA system (Phadia, Uppsala, Sweden) according to the manufacturer's instructions. In this study only patients with specific IgE levels above 0.35 kU/l; CAP class 1 (CAP: capsulated hydrophobic carrier polymer) were included.

Yeast material and growing conditions

Yeast strain BY4741 (MATa; his3 Δ 1; leu2 Δ 0; met15 Δ 0; ura3 Δ ; Euroscarf, Frankfurt am Main, Germany) was grown in YPAD medium at 30 °C. To create a profilin knock-out mutant the coding sequence of PFY1 (Acc. No. YOR122C) was amplified using the gene specific primers (5'PFY1: 5'-AGA GCT CAG GAT GTC TTG GCA AGC ATA CAC TG-3', 3'PFY1: 5'-ACT CGA GTT AGT ATT GAA CAC CAA TCA AGT GTC-3') and cloned into pYES2 (Invitrogen, Carlsbad, CA, USA) under the control of the GAL1 promoter. Protein synthesis was induced following the manufacturer's instructions with the transfer to galactose

containing medium at $OD_{600} = 0.4$. The successful expression of PFY1 was proven by IgG immunoblotting using rabbit antiserum raised against ragweed profilin (diluted 1:10000).

For the construction of $\Delta pfy1$ the PFY1 coding region was replaced by the HIS3 gene (imidazoleglycerol-phosphate dehydratase), which was amplified from plasmid pFA6aHIS3MX5 [35] by PCR with the following oligonucleotides: 5'PFY1-His: 5'- TAC ATA CAC AAC ATA AAC CCA ACT ACG ATC CGC AAA TTA TGC GTA CGT GCA GGT CGA C -3' and 3'PFY1-His: 5'-TAG GTG GTG TAT AAG GCA AGA AAA CTT TAC CTG CAT AAA TTA ATC GAT GAA TTC GAG CTC G-3' and used for transformation. Successful integration of HIS3 was verified using external primers to the genomic region deleted (5'A: 5'-GAA GAA GGG TTG CGG TCA TC-3' and 3' D: 5' AGA TGC CAG AAT AGG TGG TGT-3', 5'HIS3MX out 5'-GAA CTG TAT CCT AGC AAG-3' and 3'HIS3MX out 5'-TAA TGA CCA TCA TCG TGC-3').

Transformation of yeast

Yeast was transformed after a modified protocol from Schiestl *et al.*, [36]. The yeast cells were grown over night at 28 °C in full medium. After two centrifugation steps, and a washing step with dest. H₂O the pellet was resuspended in 100 mM lithium acetate (LiAc). Aliquots of 50 μ l were pelleted and 240 μ l 50% PEG 3350, 36 μ l 1 M LiAc, 5 μ l Carrier DNA, 1-2.5 μ l DNA and 76.5-78 μ l dest. H₂O were added. The probes were incubated at 30 °C for 30 min and 20 min at 42 °C and afterwards aliquots were plated on SCA-medium with the appropriate amino acid composition.

The transformation of the His3MX5 gene constructs was done after the Gap repair protocol according to Oldenburg *et al.*, [28]. The upstream primer 5'PFY1-His was located between 278 and 317 bp, the downstream primer 3'PFY1-His was located between 942 and 902 bp and was complementary to the chromosomal profilin gene.

Random mutagenesis

The random mutagenesis was carried out after manufacture's protocol of the GeneMorph®II Random Mutagenesis Kit (Agilent, Santa Clara, USA).

Depending on the amount of template DNA, mutation rates of 1-6 per kb can be achieved in the error-prone PCR. To get the highest number of mutations, 100 ng template DNA were added for PCR amplification. We used the plasmid p425P_{PFY}Lyc e 1.02R33 as template to avoid the complementation of non-mutated Lyc e 1.02 in Δ pf_n yeast cells. The primers were specific uM1 (5'-GAG AAA AGA TAA ATT ACA TAC ACA ACA TAA ACC CAA-3') and uM2 (5'-GCG TGA ATG TAA GCG TGA CAT AAC TAA TTA CAT GAC-3'), located -35 to -17 and +432 to 456 of the ATG of Lyc e 1.02.

Protein expression in yeast cells

The expression of recombinant profilin was induced by activation of the GAL1-promoter. One single colony was grown over night in 15 ml of SCA-glucose medium. The OD₆₀₀ was determined and the cells transferred into induction medium and incubated at 30 °C. Samples were taken after 0, 2, 4, 6, 8, 10 and 24 hours after induction mixed with SDS-loading buffer and analyzed with SDS-PAGE.

Preparation of yeast protein extracts and western blotting

For Western Blotting, yeast cells were lysed by means of glass beads (Ø 0,45 mm) in 25 mM HEPES pH 7.5, 12 mM MgCl₂, 0.5 mM EDTA, 8 mM DTT, 0.1% (v/v) Triton X-100 in the presence of protease inhibitors (Roche) at 4 °C. Samples were cleared by centrifugation

(16.200 g, 15 min at 4 °C) and the protein content of the supernatant was determined according to the method of Bradford [37] with the BioRad protein assay solution (BioRad Laboratories, Hercules, California). An aliquot of the supernatant was immediately mixed with SDS-polyacrylamide gel electrophoresis (PAGE) loading buffer, denatured at 95 °C for 10 min and subjected to a 12.5% SDS-PAGE under reducing conditions. Recombinant Lyc e 1 was expressed and purified as described previously [18]. Separated proteins were blotted onto porablot NCL Nitrocellulose membrane (Macherey-Nagel, Düren, Germany). The blot was blocked in 5% (w/v) non-fat dry milk in Tris-buffered saline (TBS/T, 20 mM Tris-HCl, pH 7.6, 200 mM NaCl, 0.1% (v/v) Tween 20) for 1 h and afterwards incubated in 1:10 diluted patients' sera overnight. Immuno-staining of bound IgE antibodies was performed with a HRP-conjugated goat-anti-human IgE antibody (Sigma-Aldrich) in a 1:20000 dilution. Visualization was carried out using ECL chemiluminescence.

Homology modelling

Structures of Lyc e 1 profilin wild type as well as mutants R33W/I15T/H19R, R33W/N38S/I118F and R33W/T48S were modelled using the MODELLER program, version 9.10 [38]. The crystal structure of profilin I from *Arabidopsis thaliana* (PDB code 3NUL, 1.6 Å resolution, 78% sequence identity) served as template for all models. Secondary structure was assigned with the DSSP program [39].

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REFERENCES

- 1 Sicherer, S. H. and Sampson, H. A. (2010) Food allergy. *J Allergy Clin Immunol* **125**, 116-25.
- 2 Wuethrich, B. (1998) Food-induced cutaneous adverse reactions. *Allergy* **53**, 131-135.
- 3 Ebner, C., Hirschwehr, R., Bauer, L., Breiteneder, H., Valenta, R., et al. (1995) Identification of allergens in fruits and vegetables: IgE cross-reactivities with the important birch pollen allergens Bet v 1 and Bet v 2 (birch profilin). *J Allergy Clin Immunol* **95**, 962-9.
- 4 Ganglberger, E., Radauer, C., Wagner, S., Riordain, G., Bezzhold, D.H. et al. (2001) Hev b 8, the Hevea brasiliensis latex profilin, is a cross-reactive allergen of latex, plant foods and pollen. *Int Arch Allergy Immunol.* **125** (3): 216-27
- 5 Westphal, S., Kempf, W., Foetisch, K., Retzek, M., Vieths, S., et al. (2004) Tomato profilin Lyc e 1: IgE cross-reactivity and allergenic potency. *Allergy* **59**, 526-32.
- 6 Willeroider, M., Fuchs, H., Ballmer-Weber, B. K., Focke, M., Susani, M., et al. (2003) Cloning and molecular and immunological characterisation of two new food allergens, Cap a 2 and Lyc e 1, profilins from bell pepper (*Capsicum annuum*) and Tomato (*Lycopersicon esculentum*). *Int Arch Allergy Immunol* **131**, 245-55.
- 7 Yu, L. X., Nasrallah, J., Valenta, R., Parthasarathy, M. V. (1998) Molecular cloning and mRNA localization of tomato pollen profilin. *Plant Mol Biol* **36**, 699-707.
- 8 Nowak-Wegrzyn and A.Muraro, A. (2011) Food allergy therapy: is a cure within reach? *Pediatr Clin North Am* **58**, 511-30, xii.
- 9 Paschke, A. (2009) Aspects of food processing and its effect on allergen structure. *Mol Nutr Food Res* **53**, 959-62.
- 10 Scheurer, S. and Sonnewald, S. (2009) Genetic engineering of plant food with reduced allergenicity. *Front Biosci* **14**, 59-71.
- 11 Peters, S., Imani, J., Mahler, V., Foetisch, K., Kaul, S., et al. (2011) Dau c 1.01 and Dau c 1.02-silenced transgenic carrot plants show reduced allergenicity to patients with carrot allergy. *Transgenic Res* **20**, 547-56.
- 12 Le, L. Q., Lorenz, Y., Scheurer, S., Fotisch, K., Enrique, E., et al. (2006) Design of tomato fruits with reduced allergenicity by dsRNAi-mediated inhibition of ns-LTP (Lyc e 3) expression. *Plant Biotechnol J* **4**, 231-42.
- 13 Le, L. Q., Mahler, V., Lorenz, Y., Scheurer, S., Biemelt, S., et al. (2006) Reduced allergenicity of tomato fruits harvested from Lyc e 1-silenced transgenic tomato plants. *J Allergy Clin Immunol* **118**, 1176-83.
- 14 Lorenz, Y., Enrique, E., Lequynh, L., Fotisch, K., Retzek, M., et al. (2006) Skin prick tests reveal stable and heritable reduction of allergenic potency of gene-silenced tomato fruits. *J Allergy Clin Immunol* **118**, 711-8.
- 15 Giehl, K., Valenta, R., Rothkegel, M., Ronsiek, M., Mannherz, H. G., et al. (1994) Interaction of plant profilin with mammalian actin. *Eur J Biochem* **226**, 681-9.
- 16 Rothkegel, M., Mayboroda, O., Rohde, M., Wucherpennig, C., Valenta, R., et al. (1996) Plant and animal profilins are functionally equivalent and stabilize microfilaments in living animal cells. *J Cell Sci* **109** (Pt 1), 83-90.
- 17 Christensen, H. E., Ramachandran, S., Tan, C. T., Surana, U., Dong, C. H., et al. (1996) Arabidopsis profilins are functionally similar to yeast profilins: identification of a vascular bundle-specific profilin and a pollen-specific profilin. *Plant J* **10**, 269-79.

- 18 Le, L. Q., Mahler, V., Scheurer, S., Foetisch, K., Braun, Y., et al. (2010) Yeast profilin complements profilin deficiency in transgenic tomato fruits and allows development of hypoallergenic tomato fruits. *Faseb J* **24**, 4939-47.
- 19 Fedorov, A. A., Ball, T., Mahoney, N. M., Valenta, R., Almo, S. C. (1997) The molecular basis for allergen cross-reactivity: crystal structure and IgE-epitope mapping of birch pollen profilin. *Structure* **5**, 33-45.
- 20 Asturias, J. A., Gomez-Bayon, N., Arilla, M. C., Sanchez-Pulido, L., Valencia, A., et al. (2002) Molecular and structural analysis of the panallergen profilin B cell epitopes defined by monoclonal antibodies. *Int Immunol* **14**, 993-1001.
- 21 Radauer, C., Willeroider, M., Fuchs, H., Hoffmann-Sommergruber, K., Thalhamer, J., et al. (2006) Cross-reactive and species-specific immunoglobulin E epitopes of plant profilins: an experimental and structure-based analysis. *Clin Exp Allergy* **36**, 920-9.
- 22 Lopez-Torreon, G., Diaz-Perales, A., Rodriguez, J., Sanchez-Monge, R., Crespo, J. F., et al. (2007) An experimental and modeling-based approach to locate IgE epitopes of plant profilin allergens. *J Allergy Clin Immunol* **119**, 1481-8.
- 23 Tordesillas, L., Pacios, L. F., Palacin, A., Cuesta-Herranz, J., Madero, M., et al. (2010) Characterization of IgE epitopes of Cuc m 2, the major melon allergen, and their role in cross-reactivity with pollen profilins. *Clin Exp Allergy* **40**, 174-81.
- 24 Tordesillas, L., Gamboa, P., Sanz, M. L., Palacin, A., Gomez-Casado, C., et al. (2011) A mutant of the major melon allergen, Cuc m 2, with reduced IgE binding capacity is a good candidate for specific immunotherapy. *Mol Immunol* **49**, 504-11.
- 25 Cabanos, C., Tandang-Silvas, M. R., Odijk, V., Brostedt, P., Tanaka, A., et al. (2010) Expression, purification, cross-reactivity and homology modeling of peanut profilin. *Protein Expr Purif* **73**, 36-45.
- 26 Witke, W., Sutherland, J. D., Sharpe, A., Arai, M., Kwiatkowski, D. J. (2001) Profilin I is essential for cell survival and cell division in early mouse development. *Proc Natl Acad Sci U S A* **98**, 3832-6.
- 27 Mumberg, D., Muller, R., Funk, M. (1994) Regulatable promoters of *Saccharomyces cerevisiae*: comparison of transcriptional activity and their use for heterologous expression. *Nucleic Acids Res* **22**, 5767-8.
- 28 Oldenburg, K. R., Vo, K. T., Michaelis, S., Paddon, C. (1997) Recombination-mediated PCR-directed plasmid construction in vivo in yeast. *Nucleic Acids Res* **25**, 451-2.
- 29 Thorn, K. S., Christensen, H. E., Shigeta, R., Huddler, D., Shalaby, L., et al. (1997) The crystal structure of a major allergen from plants. *Structure* **5**, 19-32.
- 30 Holm, L. and Park, J. (2000) DaliLite workbench for protein structure comparison. *Bioinformatics* **16**, 566-7.
- 31 Leitner, A., Vogel, M., Radauer, C., Breiteneder, H., Stadler, B. M., et al. (1998) A mimotope defined by phage display inhibits IgE binding to the plant panallergen profilin. *Eur J Immunol* **28**, 2921-7.
- 32 Daugherty, P. S., Chen, G., Iverson, B. L., Georgiou, G. (2000) Quantitative analysis of the effect of the mutation frequency on the affinity maturation of single chain Fv antibodies. *Proc Natl Acad Sci U S A* **97**, 2029-34.
- 33 Knoll, J. E., Ramos, M. L., Zeng, Y., Holbrook, C. C., Chow, M., et al. TILLING for allergen reduction and improvement of quality traits in peanut (*Arachis hypogaea* L.). *BMC Plant Biol* **11**, 81.
- 34 Mahfouz, M. M., Li, L. (2011) TALE nucleases and next generation GM crops. *GM Crops* **2**.
- 35 Wach, A., Brachat, A., Alberti-Segui, C., Rebischung, C., Philippsen, P. (1997) Heterologous HIS3 marker and GFP reporter modules for PCR-targeting in *Saccharomyces cerevisiae*. *Yeast* **13**, 1065-75.

- 36 Schiestl, R. H. and Gietz, R. D. (1989) High efficiency transformation of intact yeast cells using single stranded nucleic acids as a carrier. *Curr Genet* **16**, 339-46.
- 37 Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248-54.
- 38 Eswar, N., Webb, B., Marti-Renom, M. A., Madhusudhan, M. S., Eramian, D., et al. (2006) Comparative protein structure modeling using Modeller. *Curr Protoc Bioinformatics* **Chapter 5**, Unit 5 6.
- 39 Kabsch, W. and Sander, C. (1983) Dictionary of protein secondary structure: pattern recognition of hydrogen-bonded and geometrical features. *Biopolymers* **22**, 2577-637.

FIGURE LEGENDS

Table 1: Characterization of patients (history, allergic symptoms, specific IgE (CAP FEIA analysis))

patient	gender	age	occupation	clinical symptoms (tomato)	clinical symptoms (birch)	SPT (tomato) MWD [mm]	SPT (birch) MWD [mm]	CAP tomato [kU/l]	CAP birch [kU/l]	CAP rBet v 2 [kU/l]
T 002	F	44	physician	OAS	RCA	6	12	3.08	134	25.7
T 045	F	32	teacher	OAS	RCA	6	3	3.9	30.6	11.6
T 046	F	72	retiree	OAS	RCA	4	4	16.4	11.8	2.8
T 081	M	28	business economist	urticaria	RCA	4	6	1.21	17.1	2.4
B 009	F	35	lawyer	none	RCA	nd	4	0.9	30.7	1.9

F= female, M= male; OAS=Oral allergy syndrome, RCA= allergic rhinoconjunctivitis, nd= not done

Legend: Demographic data (age, gender (male/female), clinical history and symptoms of tomato allergic (T) or birch allergic (B) are displayed.

SUPPORTING INFORMATION

Supplemental Figure 1: Western immunoblot analysis of PFY1. 25 µg of crude yeast extracts were separated by SDS-PAGE. Hybridization was performed using a cross-reactive anti-ragweed profilin antibody. Expression of PFY1 was analysed over a time period of 24 h. Arrow heads indicate protein standards of 15 and 10 kDa.

Supplemental Figure 2: Analysis of profilin deficiency in the Δ pfy1 yeast. **A)** BY4741/pYES2-PFY1 yeast was transformed with the short flanking homology PCR product His3. The positive transformants were able to grow on SCA-ura-his medium (left). In case of the transformation control (BY4741/pYES2/ Δ pfy1) no growth could be observed on SCA-ura-his medium (right). **B)** Genomic DNA of WT and Δ pfy1 yeast was isolated and tested via PCR. PCR products were generated with following primer pairs: lane 1: primers 5'A and 3'D; lane 2: primers 5'A and 3'HIS3MX; lane 3: primers 5'HIS3MX and 3'D. The Arrow heads indicate DNA standards of 1500 bp and 750 bp.

Supplemental Figure 3: Growth analysis of Δ pfy1. **A)** The profilin deficient yeast strain was co-transformed with 0.5 µg Lyc e 1.02_{mut} and 0.5 µg linearized vector p425P_{PFY} and incubated on SCA-leu-his medium. **B)** As control, Δ pfy1 yeast was transformed with 0.5 µg Lyc e 1.02 and 0.5 µg p425P_{PFY}.

Supplemental Figure 4: Structure analysis of Lyc e 1 variants. Comparison of the folding structure (homology models) of Lyc e 1.02 R33 (**A**, **C**, **E**) and obtained Lyc e 1.02 variants. **B)** Lyc e 1.02-13, **D)** Lyc e 1.02-5, **F)** Lyc e 1.02-17. For residue numbering see **Figure 3**.

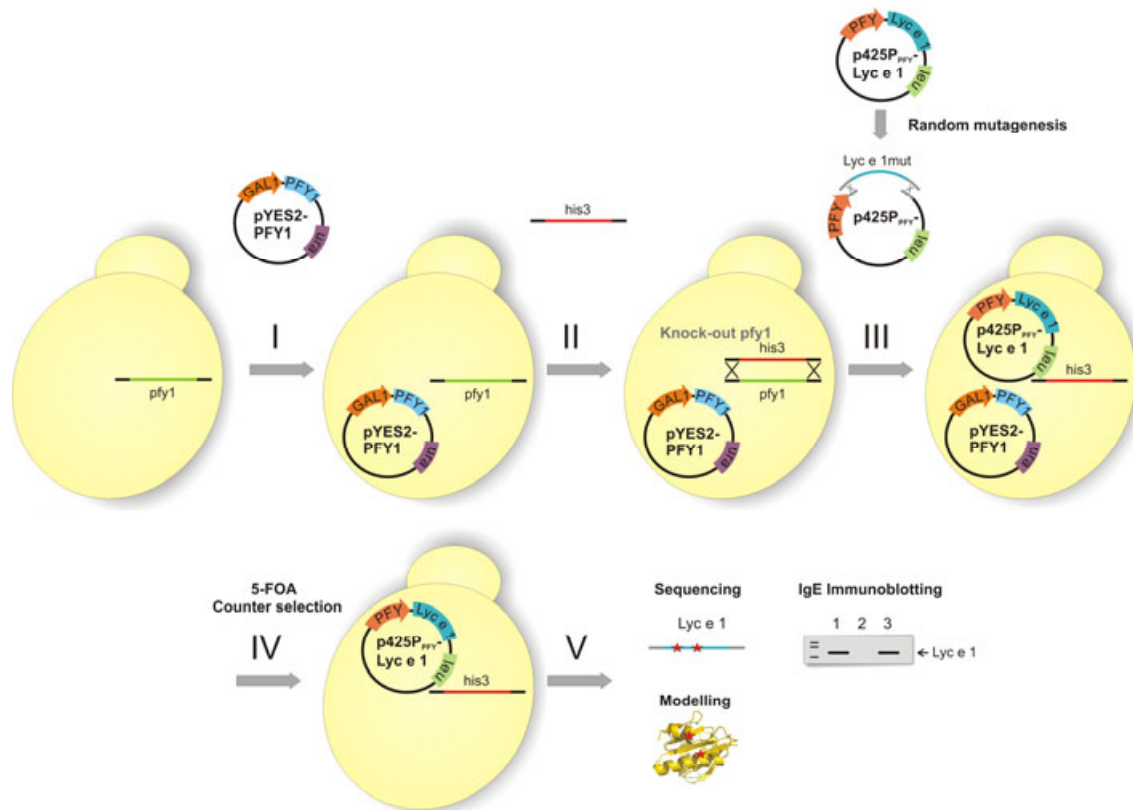


Figure 1: Strategy for the identification of functional Lyc e 1.02 variants. I) A plasmid for expression of recombinant yeast profilin was transformed into WT yeast. II) In a second transformation step, the chromosomal profilin was deleted. III) A plasmid encoding Lyc e 1.02 was randomly mutagenized to get different Lyc e 1.02 variants, the resulting PCR products were then co-transformed with linearized vector in the Δ *pfy1* knock-out yeast. IV) Afterwards the first plasmid expressing PFY1 was counter selected. V) The obtained Lyc e 1.02 variants were analysed by sequencing, modelling and IgE immunoblotting.



Figure 2: Sequence analysis of Lyc e 1 variants. Depicted is the amino acid sequence of Lyc e 1.02 (acc. no. AY061819) in black and identified non-silent amino acid changes are indicated in red.

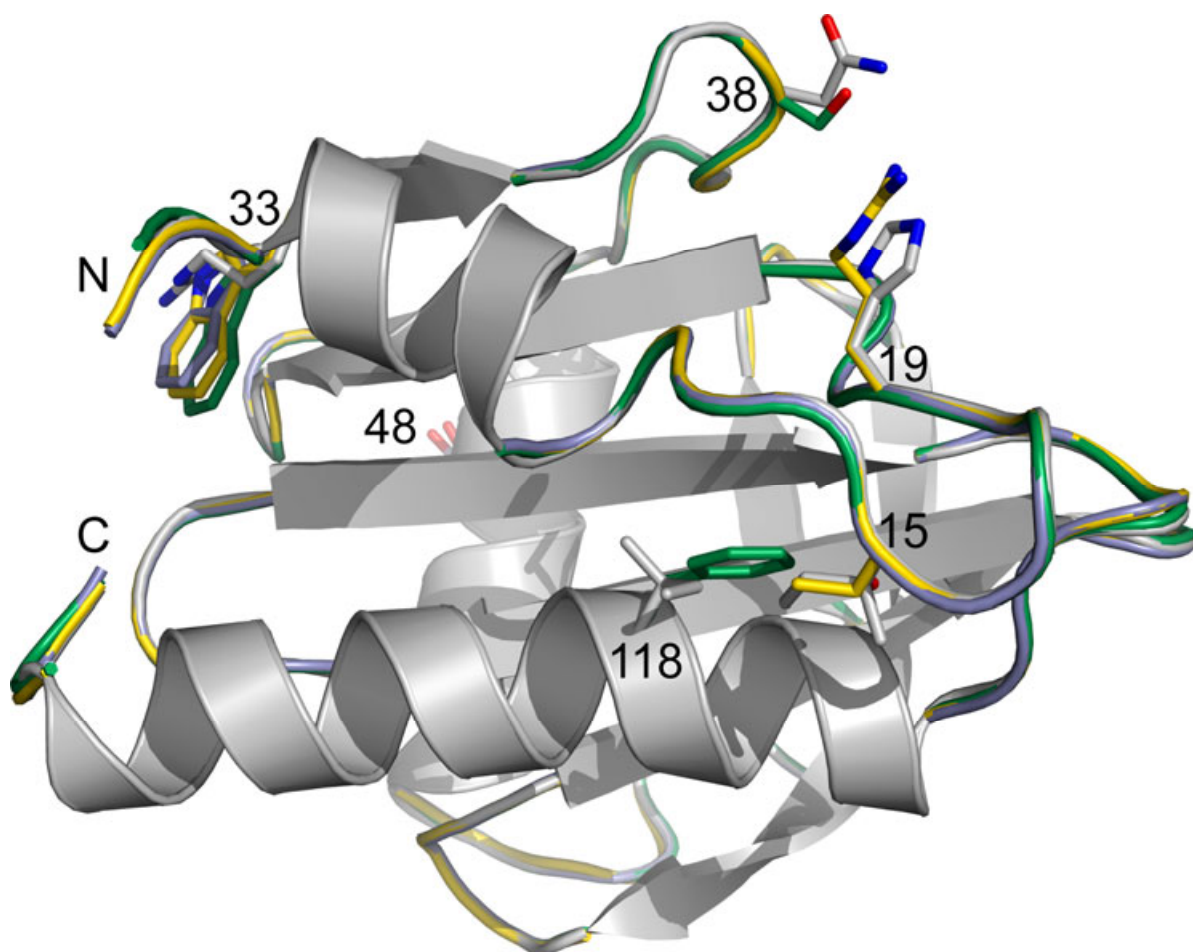


Figure 3: Structure analysis of Lyc e 1.02 variants compared with Lyc e 1.02.

Ribbon representation of superposed homology models of Lyc e 1.02 (gray) as well as mutants Lyc e 1.02-5 (yellow), Lyc e 1.02-17 (green) and Lyc e 1.02-13 (dark blue). For the mutants the main chain is only shown in the loop regions. The mutated side-chain residues are indicated in the figure. Residues M1 and L131 are labelled N and C, respectively, representing the termini of the structures. The PyMOL program was used to generate the figure (The PyMOL Molecular Graphics System, Version 1.2r1, Schrödinger, LLC.).

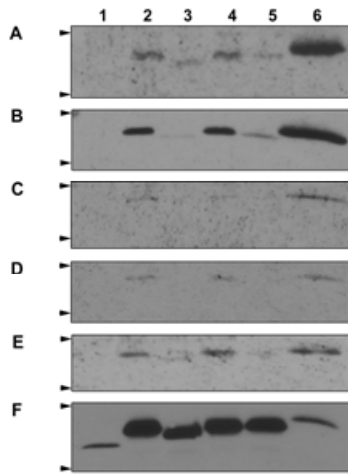
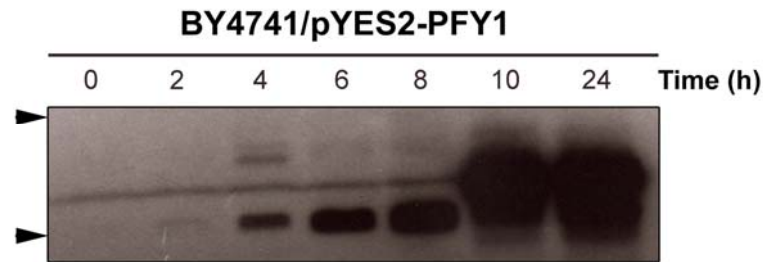


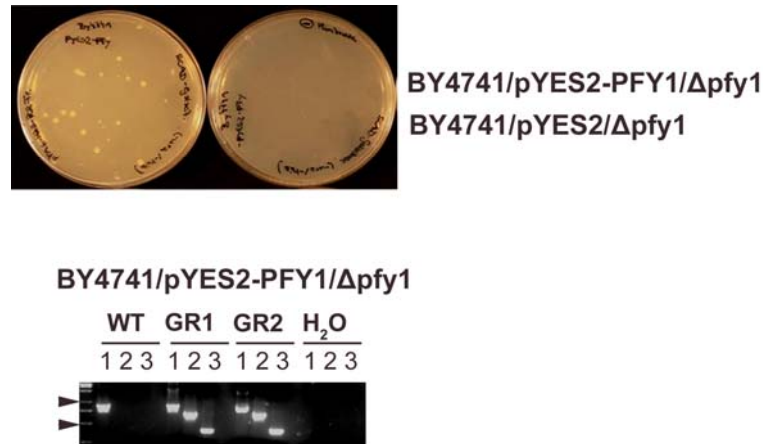
Figure 4: IgE-binding and allergenic potency of complemented Δ pfy1 yeast. Investigation of IgE (**A-E**) and IgG (**F**) binding to soluble protein extracts from crude yeast extracts of WT (lane 1), transformed control Lyc e 1.02 (lane 2), and complemented mutants Lyc e 1.02-5 (lane 3), Lyc e 1.02-13 (lane 4) and Lyc e 1.02-17 (lane 5). 0.5 μ g recombinant Lyc e 1 was loaded as positive control (lane 6). Proteins were separated on 15% SDS-PAGE, blotted to nylon membranes and probed with sera from tomato allergic patients T 002 (**A**), T 045 (**B**), T 046 (**C**), T 081 (**D**) and birch allergic patient B 009 (**E**) and rabbit-anti-ragweed profilin antiserum (**F**). Arrow heads indicate protein standards of 15 and 10 kDa, respectively.

Fig. S1



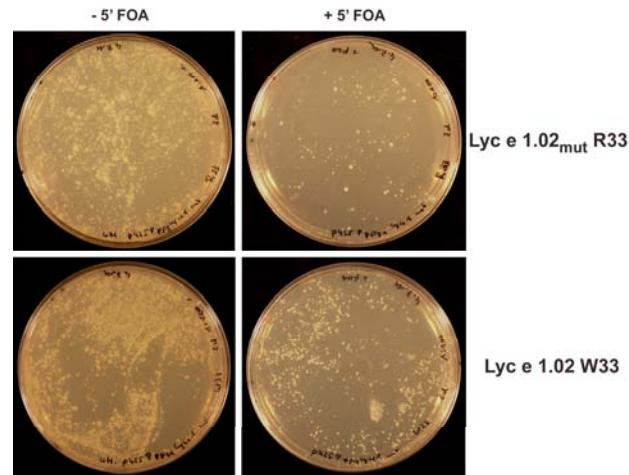
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Fig. S2



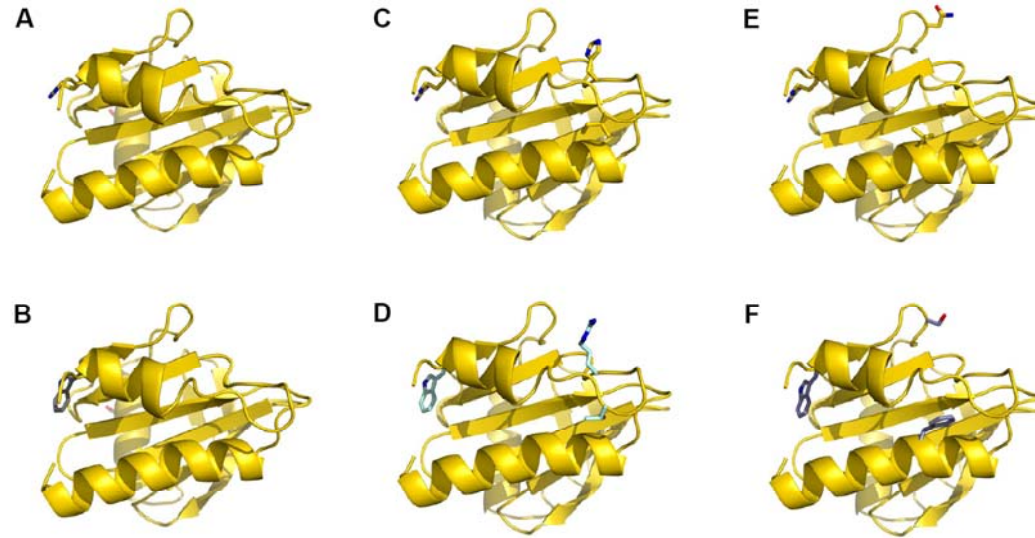
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Fig. S3



Supplemental Figure 3: Growth analysis of Δ pfy1. **A)** The profilin deficient yeast strain was co-transformed with 0.5 μ g Lyc e 1.02_{mut} and 0.5 μ g linearized vector p425P_{PFY} and incubated on SCA-leu-his medium. **B)** As control, Δ pfy1 yeast was transformed with 0.5 μ g Lyc e 1.02 and 0.5 μ g p425P_{PFY}.

Fig. S4



Supplemental Figure 4: Structure analysis of Lyc e 1 variants. Comparison of the folding structure (homology models) of Lyc e 1.02 R33 (**A**, **C**, **E**) and obtained Lyc e 1.02 variants. **B**) Lyc e 1.02-13, **D**) Lyc e 1.02-5, **F**) Lyc e 1.02-17. For residue numbering see **Figure 3**.