Expression of Zm13, a pollen specific maize protein, in *Escherichia coli* reveals IgE-binding capacity and allergenic potential

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Abstract Plant proteins belong to the most frequent elicitors of type I allergic symptoms in industrialized countries. Several relevant plant allergens have been found to be either specifically expressed or highly upregulated in mature pollen. The cDNA coding for a pollen specific maize protein, Zm13, shows significant sequence homology with a number of pollen or anther specific proteins from monocot and dicot plants as well as with recently described allergens from olive and rye grass. To test whether the Zm13 protein might possess IgE-binding capacity, Zm13 was expressed in *E. coli*. The coding region of Zm13 was PCR amplified from a genomic clone and expressed as as a glutathione-*S*-transferase fusion protein. The recombinant Zm13 fusion protein bound a Zm13 specific rabbit antiserum and reacted with serum IgE from grass pollen allergic patients indicating that Zm13 and homologous proteins represent a family of conserved plant allergens.

Key words: Type I allergy; Pollen specific proteins; Expression in *E. coli*; IgE-binding

1. Introduction

Plant and in particular pollen-derived proteins represent potent elicitors of type I allergy in man. Highly upregulated or exclusive expression in pollen versus somatic plant tissues was found to be a major characteristic of many plant allergens [1]. Tree pollen allergens (Bet v1 [2], Bet v2 [3,4], Bet v3 [5]), grass pollen allergens (Lol p1 [6], Phl p2 [7] and Lol p5 [8]) and weed allergens such as Amb a1 [9] showed highly upregulated expression in pollen. While allergens related to group 1, 2 and 5 grass pollen allergens could not be detected in somatic plant tissues, small amounts of Bet v1 and Bet v2 homologous proteins were identified in plant derived food (fruits, vegetables and spices) as elicitors of IgE-mediated food intolerance [10–14].Among pollen specific proteins two maize proteins showed significant sequence homologies to pollen allergens from different plant species. Zm58 was shown to be highly homlogous to the Amb a1/2 family of ragweed allergens [15] and Zm13 [16,17] displayed sequence similarity with the major allergen of olive pollen, Ole e1 [18–20], and a rye grass allergen (*Lolium perenne*), Lol p11 [21].

In the present study it was investigated whether as a consequence of sequence similarity Zm13 may possess IgE-binding capacity and allergenic potential as was described for the homologous allergens from olive and rye grass. The Zm13 coding region was expressed as GST fusion protein in *E. coli*. A recombinant protein was obtained which bound a rabbit antiserum raised against a Zm13 peptide and which reacted with serum IgE from grass pollen allergic patients. These data support the concept that Zm13 and homologous plant proteins can act as wide spread allergens.

2. Materials and methods

2.1. Biological materials

Pollen from timothy grass (*Phleum pratense*) was purchased from Allergon, AB, Välinge, Sweden. Sera from 72 grass pollen allergic individuals were characterized by positive case history, skin prick test and RAST as described [22]. A rabbit antiserum raised against a synthetic peptide (amino acids 27–42: ADDPNLPDYVIQGR) as deduced from the amino acid sequence of Zm13 [16] was prepared by immunizing a rabbit with the KLH coupled peptide using Freund's adjuvant [23]. The antiserum was purified by affinity chromatography with the synthetic peptide coupled to CNBr activated Sepharose-4B [24] and recognizes the natural Zm13 protein (D.A. Crone and J.P. Mascarenhas, unpublished results). The genomic clone containing the complete coding region of Zm13 without introns is described [17]. *E. coli* strain Xl-1 Blue: *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*^{q}ZΔM15 Tn*10* (Tet^{r})] was obtained from Stratagene, La Jolla, CA and *E. coli* LE392: F^{-} *hsd*R514(rk^{-}mk^{-}) *sup*E44*sup*F58*lac*Y1 or Δ(*lac*IZY) 6*gal*K2*gal*T 22*met*B1*trp*R55l^{-} is described [25]. Plasmid pGEX-5T which directs the synthesis of a fusion protein with a histidine-hexapeptide and glutathione-*S*-transferase at its N-terminus and the recombinant protein at its C-terminus is described [26].

2.2. Hybridization of the Zm13 DNA with RNA from timothy grass pollen

Timothy grass (*Phleum pratense*) pollen RNA was isolated as described [27]. Total RNA was further purified by CsCl density centrifugation [28]. Approximately 20 μg total RNA were separated by denaturing agarose gel electrophoresis and blotted onto nitrocellulose [29]. A 16S–23S *E. coli* RNA (Boehringer, Mannheim, Germany) was used as marker. The RNA blot was hybridized with the Zm13 DNA fragment excised with *Hin*dIII and *Eco*RI from Zmg13/pBS-54 [17]. The Zm13 fragment was labelled with [^{32}P]dCTP (NEN, Stevenage, UK) by the random hexamer method [30] using a Prime a Gene system (Promega, Madison, WI, USA). Final washing conditions of the blot were 1.5×SSC, 0.1% SDS, 40°C.

2.3. Expression of Zm 13 in E. coli as a GST fusion protein

To express Zm13 in plasmid pGEX-5T the coding region was PCR amplified using Zmg13/pBS-54 as a template and the following oligonucleotides: 5′-AGA GAG AGG GATCCA TGG CCT CGG TTC CGG CTC CG-3′ and 5′-GAC GAC GAATTC TTA CTG GTC GTC GTC GTC GTC CGA-3′ (Pharmacia Biotech, Uppsala, Sweden). The primers contained a *Bam*HI and an *Eco*RI restriction site to allow unidirectional in-frame insertion of the fragment into plasmid pGEX-5T. The PCR product was digested with *Bam*HI and *Eco*RI, purified using a nick column (Pharmacia, Uppsala, Sweden) and ligated into plasmid pGEX-5T. The ligation product was transformed into *E. coli* LE392 using the calcium chloride method and colonies binding IgE antibodies were identified by immuno-screening with serum IgE from a patient with multivalent sensitivity to pollen and plant proteins [2]. Clone 14 containing the Zm13 DNA which had bound

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          1                                                  50
zmg13     MASVPAPATT  TAAVILC..L  CVVLSCAAAD  DPNLPD..YV  IQGRVYCDTC
riceAC    MASLR.....  TIPVIFGILF  YVLASTATAT  DA..PD..YV  VQGRVYCDTC
LolpXI    ..........  ..........  ..........  .DKGPG..FV  VTGRVYCDPC
at        ..........  .AKLVMLLVL  CILPAIVAAR  RGNIGKNTMV  VQGSTYCDTC
lilacEB   ..........  ..........  ......EDVP  QPPIPQ..FH  IQGQVYCDTC
lat52     .........M  AKAIVLLSAL  CILALANFA.  HCRPEV..FD  VEGKVYCDTC
OleI      ..........  ..........  ......EDIP  QPPVSQ..FH  IQGQVYCDTC

          51                                                 100
zmg13     RAGFVT.NVT  EYIAGAKVRL  ECKHFGTGKL  ERAIDGVTDA  TGTYTIELKD
riceAC    RAEFET.NVT  EYIKGAKVRL  ECKHFGTDKV  ERAIDGVTDE  TGTYKIELKD
LolpXI    RAGFET.NVS  HNVEGATVAV  DCRPFDGGES  KLKAEATTDK  DGWYKIEIDQ
at        KFGFETPESS  YFIPGATVKL  SCKDRKTXEE  VYTDKAVSDK  EGKYKFIVHD
lilacEB   RARFIT.ELS  EFIPGASIRL  QCKDRENGKI  TFTEIGYTRA  EGLYSMLVEG
lat52     RVQFET.KLS  ENLEGATVKL  QCRNISTEAE  TFSVEGVTDK  DGKYKLTVNG
OleI      RAGFIT.ELS  EFIPGASLRL  QCKDKENGDV  TFTEVGYTRA  EGLYSMLVER

          101                                                150
zmg13     SHEEDICQVV  LVASPRKDCD  EVQALRDRAG  VLLTRNVGIS  .DSLRPANPL
riceAC    SHEEDICEVV  LVHSPLANCS  EIEAERDRAR  VLLTRNVGIC  .DNLRLANPL
LolpXI    DHQEEICEVV  LAKSPDKSCS  EIEEFRDRAR  VPLTSNXGIK  QQGIRYANPI
at        DHRDQMCDVL  LVKSSDKTCS  KISVGREKSR  VILNHYSGIA  RRSDML....
lilacEB   DHKNEFCEIT  LISSGREDCD  EIPVE.GWAK  PSLKFKLNTV  NGTTRTINPI
lat52     DHENDICEVT  VVKSPREDCK  ESVSGYEKAR  IECSDNVGI.  HNAVRFANPL
OleI      DHKNEFCEIT  LISSGRKDCN  EIPTE.GWAK  PSLKFKLNTV  NGTTRTVNPL

          151                                  176
zmg13     GYFKDVPLPV  CAALLKQLDS  DDDDDQ
riceAC    GYLKDYHCP.  SAALLKQFDL  ADDDNE
LolpXI    AFFRKEPLKE  CGGILQAY..  ......
at        ..........  ..........  ......
lilacEB   GFFKKEALPK  CTQVYNKLGM  YPPNM.
lat52     FFMKAESVQG  CKEALDELGL  FPLEF.
OleI      GFFKKEALPK  CAQVYNKLGM  YPPNM.
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Fig. 1. Alignment of the amino acid sequences of Zm13 (zmg13; accession number P33050) with a pollen specific rice protein (rice AC; accession number S31710), a rye grass pollen allergen (LolpXI; accession number A54002), a pollen specific protein from *Arabidopsis thaliana* (at; accession number Z25693), an allergen-like protein from lilac (lilacEB; accession number S43242), an anther specific protein from tomato (lat52; accession number P13447) and the major allergen of olive pollen (Ole e1; accession number P19963). Amino acids which are identical to Zm13 were printed bold and points indicate gaps introduced for maximal fit.

serum IgE and a rabbit anti-Zm13 antiserum was subjected to DNA sequence analysis in situ using a T7 sequencing kit (Pharmacia, Uppsala, Sweden), [^{35}S]dCTP (NEN, Stevenage, UK) and the following oligonucleotides: *pGEXrev:* 5′-GAG CTG CAT GTG TCA GAG C 3′; *DH17*: 5′ GCG ACC GCC ACC TAC ACG-3′; *DH18:* 5′-CCA CCA GCA CCA CCT GGC 3′; *ZMF*: 5′ GCA GGG CTG GCA AGC CAG G-3′; *ZMR:* 5′-AAG CTG TGA CGG TCT CCG G-3′ by primer walking [31]. *E. coli* Le392 transformed with clone 14 were grown to an optical density at 600 nm of 0.3 and IPTG (isopropyl-β-thio-D-galactopyranoside) was added to a final concentration of 1 mM for induction of protein synthesis. After 3 h culture at 37°C *E. coli* cells were harvested by centrifugation at 2000×*g* and 4°C. Pellets were stored at −20°C until analysis.

2.4. DNA sequence comparsions and prediction of antigenic sites

The amino acid sequences of Zm13 and Zm13 homologous proteins from rice, rye grass *Arabidopsis*, lilac, tomato and olive were aligned according to maximal fit by a FASTA comparison. The flexibility and antigenic index of the mature Zm13, Lol p11 and Ole e1 proteins were caculated using the MacVector program according to [32] and [33], respectively.

2.5. Dot blots, SDS-PAGE and immunoblotting

The *E. coli* pellets containing clone 14 or pGEX-5T without inserted DNA (negative control) were resuspended in phosphate buffered saline (PBS), frozen and thawed twice and homogenized with an ultraturrax (IKA, Heidelberg, Germany) to prepare protein extracts. The proteins were analyzed by 12.5% SDS-PAGE [32] and Coomassie blue staining to estimate the protein content [34,35]. According to the protein staining comparable amounts of extracts containing recombinant Zm13 GST fusion protein or GST (negative control) were dotted to nitrocellulose membranes or were separated by denaturing SDS-PAGE and electroblotted onto nitrocellulose [36]. Nitrocellulose strips containing dot-blotted or electroblotted proteins were incubated with the 1:100 diluted affinity purified Zm13 specific rabbit antiserum or with 1:10 diluted sera from grass pollen allergic patients. Membranes were blocked and washed in buffer A: 50 mmol/l sodium phosphate pH 7.5, 0.5% v/v Tween 20, 0.5% w/v bovine serum albumin, 0.05%

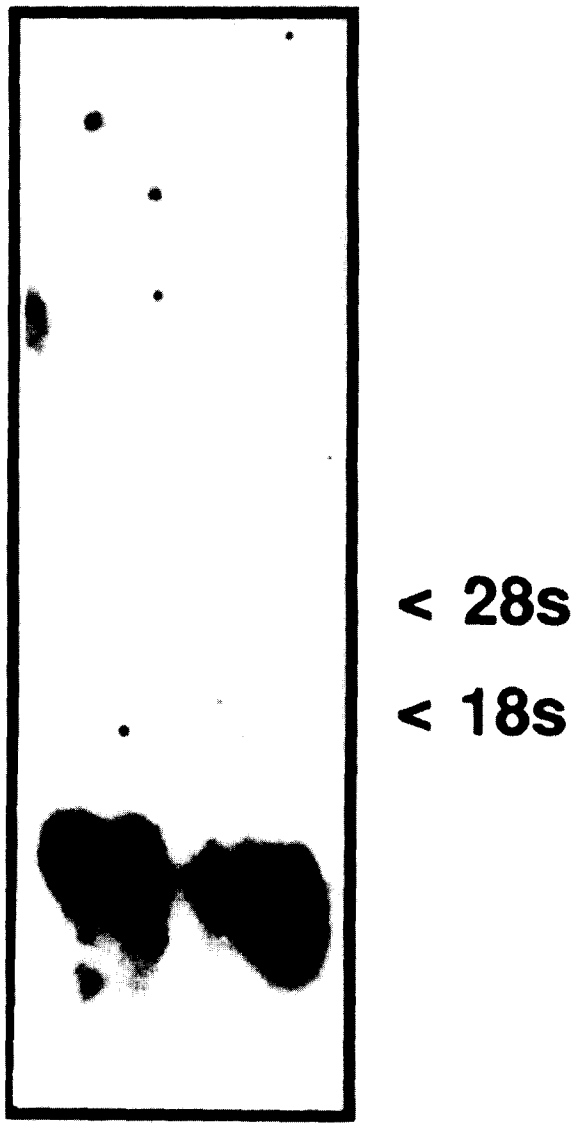

Fig. 2. Cross-hybridization of the Zm13 DNA with timothy grass (*Phleum pratense*) pollen RNA. A nitrocellulose blot containing in two lanes each approximately 20 μg of total timothy grass pollen RNA was hybridized under stringent conditions with a Zm13 cDNA probe. The position of the 28S and 18S ribosomal RNA is indicated.

w/v NaN_3 which was also used to dilute sera. Bound rabbit antibodies were detected with a 1:2000 diluted ^{125}I-labelled donkey anti-rabbit antiserum (Amersham, Buckinghamshire, UK). Bound IgE was detected with 1:10 diluted ^{125}I-labelled anti-human IgE antibodies (Pharmacia, Uppsala, Sweden) and visualized by autoradiography using Kodak X-Omat films and intensifying screens (Kodak, Heidelberg, Germany).

3. Results

3.1. Zm13 shows sequence homology and comparable antigenicity profiles with plant allergens

Fig. 1 shows that Zm13 has a significant end-to-end amino acid sequence homology with allergens from olive pollen, Ole e1 [19,20] (38% identity) and from rye grass, Lol p11 [21] (49% identity). A similar degree of sequence similarity was found with an allergen-like protein from lilac [37] and pollen specific proteins from rice [38], *Arabidopsis*, and tomato [39]. The flexibility profiles as well as the antigenicity profiles calculated for the complete mature Zm13, Ole e1 and Lol p11 proteins indicated the presence of common as well as species specific B-cell epitopes (data not shown).

3.2. The cDNA coding for Zm13 cross-hybridizes with RNA from timothy grass (Phleum pratense) pollen

The presence of Zm13 homologous transcripts in timothy grass (*Phleum pratense*) pollen representing a potent monocot allergen source was investigated by Northern blot experiments. A DNA fragment containing the complete coding region of Zm13 was ^{32}P-labelled and hybridized with nitrocellulose blotted total RNA from timothy grass pollen. Fig. 2 shows specific hybridization of the Zm13 cDNA at approximately 800–900 nucleotides. The hybridization was performed at stringent conditions (1.5×SSC, 0.1% SDS, 40°C), thus confirming the presence of a Zm13 homologous transcript in timothy grass pollen.

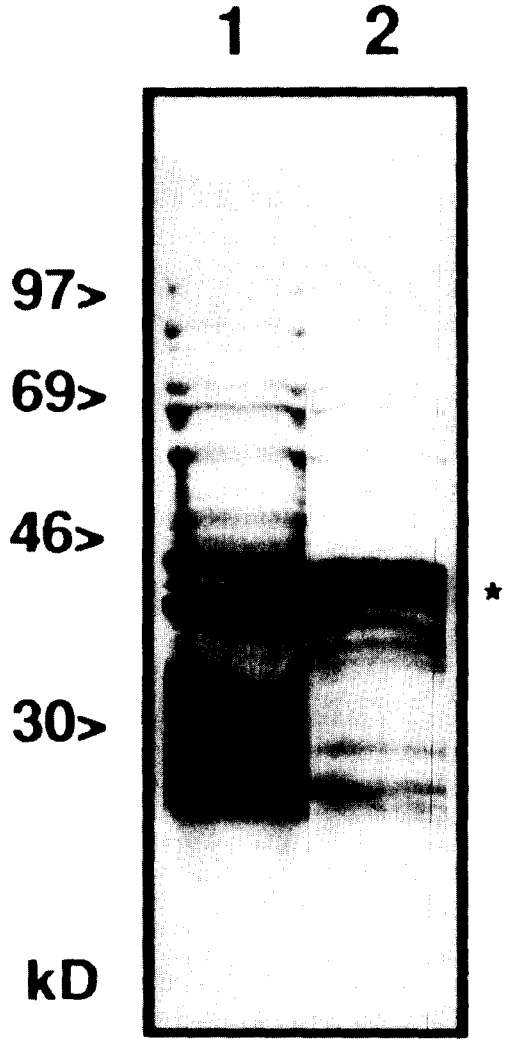

Fig. 3. Coomassie blue-stained SDS-PAGE containing GST and the Zm13 GST fusion protein. In lane 1, *E. coli* extracts containing GST and in lane 2, extracts containing the Zm13 GST fusion protein (clone 14) are shown. The asterisk indicates the position of the Zm13 GST fusion protein with a molecular weight of 44 kDa.

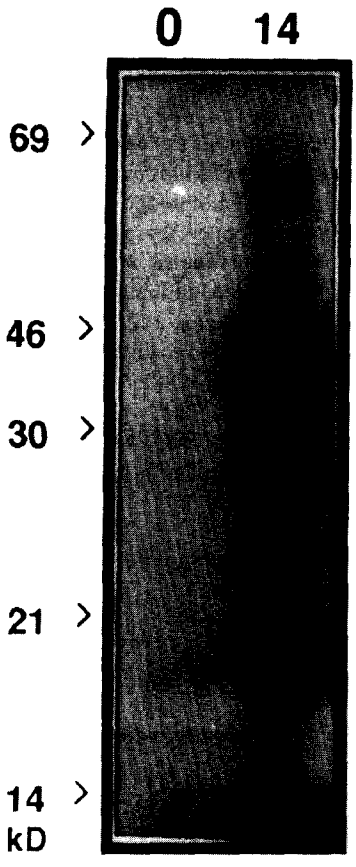

Fig. 4. A rabbit anti-Zm13 antiserum reacts with the nitrocellulose-blotted recombinant Zm13 GST fusion protein. Nitrocelluloses containing overexpressed GST (lane 0) or Zm13 GST fusion protein from clones 14 were probed with a rabbit anti-Zm13 peptide antiserum.

3.3. *Expression and immunological activity of the Zm13 GST fusion protein; recombinant Zm13 binds a Zm13 specific rabbit antiserum and allergic patient's serum IgE*

E. coli Le392 which were transformed with plasmid pGEX-5T containing the in-frame inserted Zm13 cDNA produced a 44 kDa Zm13 GST fusion upon induction with IPTG. Fig. 3 shows in lane 2 that the Zm13 GST fusion proteins represents approximately 20% of the total *E. coli* proteins (Fig. 3: lane 2; Zm13 GST fusion protein band indicated with an asterisk). When *E. coli* were transformed with the empty pGEX-5T vector, GST with a molecular weight of approximately 28 kDa was expressed (Fig. 3: lane 1; negative control).

The DNA sequence of the Zm13 fragment as inserted into pGEX-5T was determined by sequencing both strands to exclude that PCR mutations were introduced into the original sequence. In addition the Zm13 GST fusion protein was tested for reactivity with a rabbit antiserum which was raised against a synthetic peptide deduced from the Zm13 amino acid sequence. Fig. 4 shows the reactivity of the antiserum to the Zm13 fusion protein at approximately 44 kDa. Additional proteins of lower molecular weight, presumably representing degradation products were detected by the antiserum. No reactivity of the antiserum to *E. coli* transformed with the empty pGEX-5T was observed (Fig. 4: lane 0).

To investigate whether the Zm13 GST fusion protein is able to bind serum IgE-antibodies from pollen allergic individuals, we tested sera from grass pollen allergic individuals. Two types of assays were employed to measure IgE-binding to recombinant Zm13. Sera were tested with dot blotted *E. coli* extracts containing native recombinant Zm13 which was neither denatured nor reduced. In parallel, the same sera were tested with SDS-PAGE separated and nitrocellulose blotted Zm13. In both assays recombinant Zm13 showed comparable IgE-binding with 2 out of 72 sera from grass pollen allergic individuals. No significant reactivity was observed when sera from non-allergic individuals were tested or when grass pollen allergic patients were tested with *E. coli* proteins containing GST alone.

4. Discussion

Zm13 represents a protein which is specifically expressed in mature maize pollen [16,17]. Significant sequence homologies of Zm13 with other pollen or anther specific proteins have been described, but little information is available regarding the possible biological function of Zm13. Recently the major olive allergen, Ole e1, and a rye grass allergen, Lol p11, were described to be highly homologous with Zm13 [19–21]. In fact, pollen specific expression or upregulated expression in pollen tissue was found to be a cardinal feature of many plant allergens which led to the working hypothesis that pollen derived proteins and particularly proteins which are highly expressed in pollen may possess a high capacity to sensitize atopic individuals [1]. Cross-hybridization has been shown earlier as a way to verify the presence of transcripts coding for Bet v1 cross-reactive allergens in pollen of trees belonging to the order *Fagales* [40]. Using this approach the presence of Zm13 homologous transcripts in RNA from timothy grass pollen, a relevant source of monocot derived allergens, was analyzed. Even under stringent conditions a homologous transcript hybridized with the Zm13 DNA indicating that Zm13 homologues are present in grasses (timothy grass — *Phleum pratense*; rye grass — *Lolium perenne*).

High level expression of a Zm13 GST fusion protein in *E. coli* could be obtained by engineering the PCR amplified coding region of Zm13 into a pGEX-5T plasmid. Recombinant Zm13 bound a rabbit anti-Zm13 antiserum and reacted with serum IgE from 2 out of 72 grass pollen allergic individuals regardless whether the protein was tested in its native or denatured form. These data support the hypothesis that Zm13 and homologous proteins may belong to a family of conserved plant allergens, even though they are recognized by a rather low percentage of allergic patients. In fact studies estimating higher frequencies of patients' IgE reactivity with Zm13 homologous proteins from olive and grass pollen were done with purified natural proteins [41,42,21]. So far, no information was available regarding the IgE-binding capacity of recombinant Zm13 homologous allergens, tested in a representative number of allergic individuals. The rather low percentage of Zm13 reactive sera could, however, be due to the presence of IgE-epitopes outside of the conserved regions of the three proteins. A change of the Zm13 sequence by mutations introduced into the Zm13 coding sequence during the PCR amplification could be excluded by sequencing the expression construct in situ.

Another explaination for the low percentage of reactive sera may be that carbohydrate moieties which are not present on the bacterially expressed Zm13 could play a relevant role for patients IgE-binding either as primary target for IgE antibo-

dies or by maintaining proper folding of the Zm13 protein. Indeed a role of carbohydrate moieties for the IgE interaction has been discussed for Lol p11 and Ole e1 [21,43] and amino acids 57–59 (NVT) of Zm13 represent a typical *N*-glycosilation site. Such as the family of profilins [4], Zm13 and homologous allergens may be important for patients suffering from multivalent plant allergies.

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