Primary Identification, Biochemical Characterization, and Immunologic Properties of the Allergenic Pollen Cyclophilin Cat r 1

Debajyoti Ghosh, Geoffrey A. Mueller, Gabriele Schramm, Lori L. Edwards, Arnd Petersen, Robert E. London, Helmut Haas, and Swati Gupta Bhattacharya

From the 1Bose Institute, 93/1 APC Road, Kolkata 700009, India, the 2Laboratory of Structural Biology, NIEHS, National Institutes of Health, Research Triangle Park, North Carolina 27709, the 3Research Centre Borstel, Leibniz Centre for Medicine and Biosciences, D-23845 Borstel, Germany, and the 4Division of Clinical and Molecular Allergology, Research Center Borstel, Airway Research Center North (ARCN), Member of the German Centre for Lung Research, Parkallee 22, D-23845 Borstel, Germany

Background: Plant cyclophilin allergens are not well characterized. Results: Cat r 1, an allergenic pollen cyclophilin cross-reactive to fungal cyclophilins, was immunologically and biochemically characterized. An NMR structure identified a protein surface conserved between Cat r 1, fungal, and animal cyclophilins. Conclusion: IgE-mediated cross-reactivity between plant and fungal cyclophilins can be explained by their structures. Significance: This information is useful for component-resolved diagnosis and allergen immunotherapy.

Cyclophilin (Cyp) allergens are considered pan-allergens due to frequently reported cross-reactivity. In addition to well studied fungal Cyps, a number of plant Cyps were identified as allergens (e.g. Bet v 7 from birch pollen, Cat r 1 from periwinkle pollen). However, there are conflicting data regarding their antigenic/allergenic cross-reactivity, with no plant Cyp allergen structures available for comparison. Because amino acid residues are fairly conserved between plant and fungal Cyps, it is particularly interesting to check whether they can cross-react. Cat r 1 was identified by immunoblotting using allergic patients' sera followed by N-terminal sequencing. Cat r 1 (~91% sequence identity to Bet v 7) was cloned from a cDNA library and expressed in Escherichia coli. Recombinant Cat r 1 was utilized to confirm peptidyl-prolyl cis-trans-isomerase (PPIase) activity by a PPIase assay and the allergenic property by an IgE-specific immunoblotting and rat basophil leukemia cell (RBL-SX38) mediator release assay. Inhibition-ELISA showed cross-reactive binding of serum IgE from Cat r 1-allergic individuals to fungal allergenic Cyps Asp f 11 and Mal a s 6. The molecular structure of Cat r 1 was determined by NMR spectroscopy. The antigenic surface was examined in relation to its plant, animal, and fungal homologues. The structure revealed a typical cyclophilin fold consisting of a compact β-barrel made up of seven anti-parallel β-strands along with two surrounding α-helices.

This is the first structure of an allergenic plant Cyp revealing high conservation of the antigenic surface particularly near the PPIase active site, which supports the pronounced cross-reactivity among Cyps from various sources.

Cyclophilins (Cyps) were discovered as intracellular target proteins for the immunosuppressive drug cyclosporin (1). They perform a wide array of biological functions, such as catalysis of peptidyl-prolyl cis-trans-isomerization (PPIase) and as molecular chaperones (2). Structural and immunologic properties of allergenic fungal Cyps have been extensively studied (3–6). Cyps are major allergens in Aspergillus, Malassezia, and the hallucinogenic fungus Psilocybe cubensis (5–7). Sera obtained from allergic broncho-pulmonary aspergillosis patients contain IgE that specifically recognizes Aspergillus proteins, including Cyps (5). The Cyp Mal a s 6 is the major allergen produced by Malassezia, a monophyletic fungal genus found on the skin of 7 billion humans and associated with a variety of conditions, such as dandruff, atopic dermatitis, pityriasis versicolor, seborrheic dermatitis, and folliculitis (5, 8). Fungal Cyps often show IgE-mediated cross-reactivity to human Cyps, which can contribute to severity and perpetuation of allergic diseases (9).

In plants, a Cyp allergen was first identified nearly 20 years ago from birch pollen extract prepared in neutral to alkaline extraction conditions (pH 7.5–8.5) (10). This pollen allergen was later purified, characterized, cloned, and named as Bet v 7 (11, 12). Subsequently, allergenic Cyps from carrot, pumpkin, raspberry, periwinkle pollen, and rye grass pollen have been identified (11–16). Although the total number of currently identified Cyp allergens is limited, a large population of allergic subjects is potentially sensitized to Cyps from fungi and plants.

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† Supported by a fellowship from the German Academic Exchange Service (DAAD). To whom correspondence may be addressed: Division of Allergy, Immunology and Rheumatology, Dept. of Internal Medicine, University of Cincinnati College of Medicine, Cincinnati, OH 45267. E-mail: dghosh@yahoo.com.

‡ To whom correspondence may be addressed. Tel.: 913323031131; Fax 913323506790; E-mail: swati@jcbose.ac.in.

§ The abbreviations used are: Cyp, cyclophilin; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; PPIase, peptidyl-prolyl cis-trans-isomerase; RBL, rat basophil leukemia; NP-HSA, 4-hydroxy-3-nitrophenylacetyl conjugated to human serum albumin.
Thus far, only two pollen Cyps (Bet v 7 (from birch) and Cat r 1 (from *Catharanthus roseus*; trivial name: rosy periwinkle)) have been recognized as allergens in the IUIS (International Union of Immunologic Nomenclature/Allergen Nomenclature Subcommittee) database. Birch pollen is a major aeroallergen in northern Europe, whereas periwinkle abundantly grows in tropical/subtropical countries and is cultivated as a source of medicinal alkaloids. Bet v 7 was cloned in 2006, but there is no clinical/biochemical information available regarding Cat r 1. Aerobiological studies in subtropical Asia (eastern India) demonstrated airborne *C. roseus* pollen contributing up to 5% of total aero-pollen load, and extracts of *C. roseus* pollen showed a positive skin reaction in about 30% of the atopic subjects tested (17–19). Expression of Cyp in pollen is enhanced under unfavorable environmental conditions (20).

One important feature of Cyp allergens is wide ranging cross-reactivity, designating them as pan-allergens (9). However, whether plant Cyps cross-react among themselves or with human/fungal Cyps is highly debated. Fujita et al. (13) reported that there is no cross-reactivity between carrot Cyp and Bet v 7. Cadot et al. (12) demonstrated IgE-mediated cross-reactivity between Bet v 7 and other plant Cyps but no/limited cross-reactive antigenic surface and structure-based epitope on plant Cyp allergens, which is necessary for analyzing cross-reactive antigenic surface and structure-based epitope prediction.

Herein, we report the structural and immunologic properties of a plant Cyp allergen, Cat r 1 (showing >91% sequence identity with Bet v 7), for the first time and also provide evidence for wide ranging cross-reactivity between plant and fungal Cyps.

**EXPERIMENTAL PROCEDURES**

*Preparation of C. roseus Pollen Extract*—Protein from *C. roseus* pollen was extracted in 1:10 (w/v) phosphate buffer, pH 7.5, at 4 °C with gentle agitation for 4 h.

*Collection of Patients’ Sera and Healthy Control Sera—C. roseus* pollen allergens were identified using sera collected from patients (*n* = 15) visiting the outpatient department of the Allergy Clinic of the Institute of Child Health, Kolkata (India), with approval of the Institutional Ethical Committee. Donors were living in environments and/or maintaining gardens where *C. roseus* was one of the most dominant herbs. The atopic phenotype was confirmed by clinical history. Sensitization to *Catharanthus* pollen was confirmed by a well developed wheal-and-flare reaction in the skin prick tests and a confirmed history of respiratory allergy to *Catharanthus* pollen. Normal sera (*n* = 5) were obtained from healthy donors with no history or symptoms of atopy.

*IgE-specific Western Blotting—SDS-PAGE-separated proteins (12%, reducing) were transferred onto a nitrocellulose membrane (Schleicher & Schuell), blocked, cut into strips of 0.3-cm width, and incubated with individual serum samples (1:20 in TBST) collected from patients showing a positive skin reaction to *C. roseus* pollen antigen. The membrane strips were washed with TBST, and bound IgE was detected using alkaline phosphatase-conjugated monoclonal anti-human IgE (1:2000) (Allergopharma KG, Reinbek, Germany).

**N-terminal Sequencing and Preparation of a Labeled Oligonucleotide Probe**—The N terminus of an 18-kDa protein, recognized by most of the sera, was sequenced by Edman degradation as described elsewhere (22). Briefly, after electrophoresis, the proteins were transferred onto PVDF membrane (Millipore, Eschborn, Germany), using 200 mM CAPS (Sigma) buffer, pH 11.0. The membrane was washed briefly in milliQ water, and one part of it was blocked and used for immunoblotting. The other part was stained briefly in 0.1% (w/v) Coomassie Brilliant Blue R-250 in 50% methanol, destained afterward in 50% methanol, and air-dried. The 18 kDa band was excised and microsequenced by using a protein sequencer with an online phenylthiohydantoin-derivative analyzer (Prociuse, Applied Biosystems, Weiterstadt, Germany).

The deduced cDNA information was used to design an oligonucleotide probe (5′-CCT AGA GTT TTC TTC GAT ATG AGC-3′), which was synthesized commercially (MWG Biotech AG, Germany) and labeled at the 3′-end using digoxigenin-ddUTP (DIG oligonucleotide 3′-end labeling kit, Roche Applied Science), according to the manufacturer’s instructions.

*Screening of C. roseus cDNA Library and in Vivo Excision—The C. roseus cDNA library in Lambda ZAP-II (Stratagene, La Jolla, CA) was kindly provided by Prof. Johan Memelink (University of Leiden, The Netherlands). Hybridization was done on Hybond-N+ nylon membranes (Amer sham Biosciences) incubated for 18 h at 50 °C. Four clones were isolated. Purified phage clones were used for *in vivo* excision according to the manufacturer’s protocol. The rescued plasmids from all of the clones were isolated and sequenced.

**Expression and Purification of Recombinant Cat r 1**—The relevant cDNA was subcloned into the high expression vector His-tag vector pProExHTb (Invitrogen) following amplification by PCR. Therefore, two primers (sense, 5′-ATA TAT TAT GGC GCC CCT AAC CCT AGA GTT TTC TTC GAT ATG AGC-3′; antisense, 5′-ATA TAT AAG CTT TCA GAA AGT ACC ACC AAT CTC-3′; restriction sites of KasI and HindIII are underlined) were synthesized, and after amplification, the cDNA fragment was ligated into the high expression vector and electroporated into *Escherichia coli* DH5α cells.

Transformed *E. coli* cells were grown at 37 °C up to an *A*_{600} = 0.7, and the protein was induced by β-1,3-thiogalactopyranoside (final concentration of 0.6 mM) and purified by Ni^{2+} affinity chromatography. For all subsequent experiments requiring larger amounts of protein (*i.e.* NMR spectroscopy), the cDNA encoding Cat r 1 was codon-optimized for *E. coli*, commercially synthesized (Genscript Inc.), and inserted into a PET-21 vector, followed by protein induction and purification as described above.

**Rat Basophil Leukemic (RBL) Cell β-Hexosaminidase Assay**—A mediator release assay was performed (to confirm allergic activity of the protein used for downstream analysis) as described previously, with minor modifications (23). Briefly, RBL cells stably expressing human FcεRI (RBL-SX38 cell line obtained from Dr. J. P. Kinet and Dr. M.-H. Jouvin, Harvard
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Medical School) were cultured in complete Eagle’s minimum essential medium supplemented with 100 µg/ml G-418, and their FcεRI expression was verified by flow cytometry using anti-FcεRI (clone 3G6, Millipore/Upstate) and phycoerythrin-labeled secondary antibody. For all experiments, RBL cells were cultured in the absence of G-418. Confluent monolayers of cells were incubated overnight with 1:10 diluted sera obtained from patients or normal control subjects. Cells were then washed and incubated with Cat r 1 (10 µg/ml) diluted in triggering buffer (1% BSA dissolved in Tyrode solution, pH 7.4; Sigma), for 30 min. For determining total mediator release, cells were treated (1% BSA dissolved in Tyrode solution, pH 7.4; Sigma), for 30 min. For determining total mediator release, cells were treated (1% BSA dissolved in Tyrode solution, pH 7.4; Sigma), for 30 min.

10 C. roseus direct ELISA to detect the presence of serum IgE against positive serum samples were further evaluated by IgE-specific Cross-reactivity between Cat r 1 and Fungal Cyps —Skin test-positive serum samples were further evaluated by IgE-specific direct ELISA to detect the presence of serum IgE against C. roseus pollen extract as described previously (28). Briefly, polystyrene microtiter plates (Corning, Inc.) were coated with 10 µg/ml Cat r 1 in 0.2 M carbonate-bicarbonate buffer (pH 9.4) at 4 °C overnight. Following coating, the plates were washed four times for 5 min each with PBS containing 0.1% Tween 20 (PBS-T), followed by blocking with 100 µl of 2% bovine serum albumin (Sigma) in PBS-T for 3 h. After washing with PBS-T, patients’ sera diluted (1:10) with blocking solution were added and incubated for 16 h, followed by washing as above. Then 100 µl of goat anti-human IgE (KPL) diluted 1000 times in blocking solution was added and incubated at room temperature for 3 h, washed again, and detected with rabbit anti-goat IgG-alkaline phosphatase conjugate (KPL) using paranitrophenyl phosphate substrate (Sigma). The result was expressed as values obtained using patient serum/average OD value obtained using normal control sera (P/N values). Total IgE values of all sera were estimated using the Pathozyme total IgE quantification kit (Omega Diagnostics, Alva, UK), as described previously (29).

ELISA inhibition was done to assess cross-reactivity between Cat r 1 and fugal Cyp allergens as described elsewhere (28, 29). Briefly, four serum samples with Cat r 1-specific P/N values of >4 were selected, pooled, and preincubated overnight at 4 °C with equal volumes of serially diluted Cat r 1, Asp f 11, and Mala s 6, respectively. The latter two fungal Cyps were kindly provided by Dr. Reto Crameri and Dr. Sabine Flueckiger from the Swiss Institute of Allergy and Asthma Research (Davos, Switzerland). The subsequent part of the test was performed in polystyrene microtiter plates (Corning) coated with 10 µg/ml Cat r 1 (100 µl). The coated plates were washed and incubated with inhibited sera overnight at 4 °C, and the bound IgE was detected as above.

Next, we generated a codon-optimized loop deletion mutant of Cat r 1 (commercially synthesized from Genewiz Inc.) to further investigate the effect of the RSGKPLH loop, which is specifically present in plant Cyps. Both Cat r 1 and its loop deletion mutant were subcloned in pDest566 expression vector (kindly provided by Dom Esposito, National Institutes of Health) and expressed in E. coli. The folding of both the WT and mutant proteins was checked by circular dichroism, which showed a fairly identical pattern (data not shown). We performed immunoblots using a rabbit anti-plant Cyp antibody, kindly supplied by Dr. Charles Gasser (University of California, Davis, CA) (30, 31). In addition, we used a commercially available rabbit anti-animal Cyp antibody (Cell Signaling Technology), which recognizes Cyps from several animal species (human, murine, bovine, and canine Cyps), to check cross-reactivity to Cat r 1 and its loop deletion mutant in immunoblots.

Finally, we used two Catharanthus-sensitized patients’ sera to check serum IgE binding to the loop deletion mutant of Cat r 1 in IgE-specific immunoblots following the method described above.

CD and NMR Spectroscopy —CD measurements were performed to check the quality of the recombinant protein. Briefly, this was done in 20 mM phosphate buffer, pH 7.2, at 25 °C with a 4.2 µM protein concentration. The CD spectra were obtained on a JASCO spectropolarimeter (J-600) using a 1-mm path length cell. The far-UV CD spectrum was registered in the range of 190–250 nm. Molar ellipticity values were plotted against wavelength. The spectrum was analyzed for secondary structure content using the program CDNN (32).

A [U-13C,15N]Cat r 1 sample was expressed in E. coli. The quality of the 1H-15N HSQC spectrum was judged to be excellent. NMR chemical shift assignments were 100% complete for the backbone atoms of Cat r 1. Resonance assignments have been deposited in the BioMagResBank database, entry 19432. The solution structure was determined as described elsewhere (33). The 15N-1H HSQC spectrum of the backbone amides was unusually disperse, making the backbone assignments facile (see below). The dispersion was probably due to the high β-strand content and numerous aromatic residues (13 Phe and 2 Tyr). However, the aromatic side chains had very poor dispersion, and only half could be confidently assigned even with the assistance of the initial structures and models. However, the models did explain some chemical shifts that were more than two S.D. values from the mean despite the missing assignments. For example, the β protons of Leu-111 were 0.14 and −0.01 ppm. This is most likely due to the position of these protons in the face of an aromatic ring. The initial structures and a CS23D
was found to be present in both of the groups (indicated by a reactive protein with an approximate molecular mass of 18 kDa immunoblot using a control serum. Considering the dielectric constants of 2 and 80 for protein and r 1 surface using APBS (Adaptive Poisson-Boltzmann Solver), electrostatic potentials of the residues were mapped on the Cat LLC, New York). Stereochemical quality and the solvent-accessible surface of Cat r 1 were deduced using VADAR (38). The structure were used to map the conserved sequences on the surface of Cat r 1 using the program ConSurf, which scores the amino acids according to their degree of conservation (37). The final figure was generated using PyMOL (Schroedinger, LLC, New York). Stereochemical quality and the solvent-accessible surface of Cat r 1 were deduced using VADAR (38). The electrostatic potentials of the residues were mapped on the Cat r 1 surface using APBS (Adaptive Poisson-Boltzmann Solver), considering the dielectric constants of 2 and 80 for protein and solvent, respectively, and an ionic strength of 0.3 M, as described previously (39, 40).

RESULTS

Identification and cDNA Cloning of the 18-kDa Allergen; Characterization as a Cyp—IgE-specific Immunoblotting using sera obtained from Catharanthus-allergic patients showed at least two distinct patterns (Fig. 1). Whereas one group of sera reacted with several components (lanes 3, 4, 7, and 8), the other group recognized only two protein bands. An IgE-reactive protein with an approximate molecular mass of 18 kDa was found to be present in both of the groups (indicated by a red arrow in Fig. 1). Interestingly, periodate treatment of blotted C. roseus pollen caused a considerable decrease of IgE reactivity to all of the detected bands except for the 18 kDa band (data not shown), indicating that the 18 kDa band is probably a non-glycosylated protein (41). Clinical features of serum donors identified by history, positive skin test, and IgE-specific ELISA are summarized in Table 1.

N-terminal sequencing of the band revealed the clear sequence PNPRVFFDMSVGG, corresponding to cytosolic Cyp. This was used to design a sequence-specific 24-mer oligonucleotide probe to screen the cDNA library of C. roseus. We obtained four clones. The clones were sequenced and found to have identical protein-coding regions. Fig. 2 shows the cDNA and deduced amino acid sequence (submitted to GenBankTM, accession number JF 973325). The gene sequence was found to be identical to cytosolic Cyp sequence of periwinkle deposited in the Plant Gene Register (GenBankTM X85185) (42). The deduced amino acid sequence also showed considerable sequence identity to allergenic Cyps, namely Asp f 11 from Aspergillus fumigatus, Mala s 6 from Malassezia sympodiales, and human CYP A (58, 65, and 71%, respectively).

The recombinant protein (named Cat r 1, following the IUIS allergen nomenclature system) was expressed in E. coli as His6-tagged protein and purified using a Ni2+-nitrilotriacetic acid column. It was found to be sufficiently pure because no other contaminating bands were observed in Coomassie-stained denaturing SDS-PAGE (Fig. 3a, lane 1). Recombinant Cat r 1 was recognized by IgE from patients’ sera and thus retained allergenic activity (Fig. 3a, lanes 2 and 3). RBL-SX38 cells presensitized with individual patients’ sera showed variable degrees of β-hexosaminidase release when exposed to 10 μg/ml Cat r 1 (Fig. 3b). This release accounted for 12–28% of total β-hexosaminidase, whereas cross-linking using NP-HSA and anti-NP IgE caused about 55% release. Spontaneous β-hexosaminidase release (medium control) and unconjugated HSA control were estimated to be less than 2% (data not shown). This further establishes the allergic relevance of the recombinant Cat r 1.

**TABLE 1**

| Patient characteristics |
|-------------------------|
|                         |
| **M, male; F, female; AR, allergic rhinitis; BA, bronchial asthma; AD: Atopic dermatitis.** |
| **Patient no.** | **Age** | **Gender** | **Symptoms** | **Total IgE** | **Specific IgE (P/N)*** |
|-------------------|---------|------------|---------------|---------------|------------------------|
| 1                 | 26      | M          | AR + AD       | 523           | 4.1                    |
| 2                 | 24      | M          | AR            | 302           | 2.8                    |
| 3                 | 21      | F          | AR            | 431           | 5.2                    |
| 4                 | 31      | M          | AR + AD       | 604           | 4.4                    |
| 5                 | 39      | M          | AR            | 240           | 3.9                    |
| 6                 | 45      | M          | AR + BA       | 442           | 2.8                    |
| 7                 | 46      | M          | AR + BA       | 590           | 5.2                    |
| 8                 | 32      | F          | AR            | 450           | 2.4                    |
| 9                 | 48      | M          | AR + BA       | 495           | 3.7                    |
| 10                | 29      | F          | AD            | 440           | 3.8                    |
| 11                | 39      | M          | AR            | 470           | 3.0                    |
| 12                | 47      | M          | AR            | 290           | 3.6                    |
| 13                | 52      | M          | AR + BA       | 490           | 4.2                    |
| 14                | 56      | F          | AR            | 330           | 2.9                    |
| 15                | 32      | M          | AR            | 406           | 4.8                    |

*The result was expressed as OD values obtained using patient serum/average value obtained using normal control sera (P/N patient’s).
FIGURE 2. Nucleotide and deduced amino acid sequence of Cat r 1. The 489 nucleotides encode a 172-amino acid protein. The N terminus of the protein, whose sequence was determined by Edman degradation, is underlined. The two potential N-linked glycosylation sites (Asn⁷⁸ and Asn¹¹⁵) are indicated in boldface type.

FIGURE 3. α, SDS-PAGE and IgE-specific immunoblot of recombinant Cat r 1. Cat r 1 expressed in E. coli was subjected to 12% SDS-PAGE (lane 1, Coomassie stain; lanes 2 and 3, IgE-specific immunoblot using two individual patients’ sera (lanes 2 and 3). β, recombinant Cat r 1 IgE-specifically triggers β-hexosaminidase release. FceR1-expressing RBL cells were incubated with individual patients’ sera (P1–P5) and, subsequently, incubated with recombinant Cat r 1. The percentage of mediator release (vertical axis) was calculated considering total mediator release from lysed cells as 100% (total). Negative controls were mediator release using control sera (Con1–3); positive controls were β-hexosaminidase release following stimulation of anti-NP IgE-sensitized RBL cells with NP-HSA. Error bars, S.D.
PPIase Activity—Fig. 4 shows the result of the enzymatic activity assay. It shows that the addition of Cat r 1 (a) caused an increase in the cleavage rate of the substrate peptide by the proteolytic enzyme in comparison with the control set (b). This higher rate of proteolytic cleavage in the presence of Cat r 1 reflects an accelerated cis to trans isomerization of the Ala-Pro peptide bond of the test peptide in the presence of Cat r 1 and, thus, indicates that the recombinant allergen is enzymatically active.

Determination of Cat r 1-specific Serum IgE and IgE-mediated Cross-reactivity—Because Cat r 1 showed high sequence identity to fungal Cyp allergens, we checked whether they exhibit IgE-mediated cross-reactivity. First, an IgE-specific ELISA was performed to check the binding of skin prick test-positive patients’ IgE to plate-bound recombinant Cat r 1. Next, a pool of sera (P/N value > 4) from five Cat r 1-allergic patients was used for an ELISA inhibition experiment with Cat r 1-coated wells and Asp f 11 and Mala s 6 as inhibitors. Both fungal allergens showed partial inhibition of IgE-binding to Cat r 1, indicating that they contain shared antigenic determinants with Cat r 1. Mala s 6 showed higher inhibition compared with Asp f 11 (Fig. 5a).

Immunoblot experiments showed that rabbit antibody raised against plant Cyp can recognize Cat r 1 and its loop deletion mutant with little/no appreciable change in band intensity. Similar results have been obtained by using the commercially derived anti-Cyp antibody, which has been demonstrated to recognize several animal Cyp proteins (Fig. 5b, lanes 3–6). No signal could be detected with BSA or secondary antibodies used as negative controls (data not shown). IgE-specific immunoblotting showed that sera from Catharanthus-sensitized patients, but not from the non-atopic control, recognized both Cat r 1 and its loop deletion mutant (Fig. 5b, lanes 7–12).

Cat r 1 Structure Determination—The secondary structure of Cat r 1 was estimated from its far UV CD spectrum (Fig. 6a). The spectrum was deconvoluted using the software CDNN, which uses a neural networking algorithm. Calculated approximate values of different structural elements are as follows: 9% α-helix, 58% β-strand, and 35% random coil, consistent with a properly folded protein. Fig. 6b shows the 1H-15N HSQC spectrum of [U-13C-15N]Cat r 1. The peaks are nicely dispersed and fairly uniform in intensity.

An initial model of Cat r 1 was created using the backbone assignments and chemical shift/homology modeling program CS23D (34). The model facilitated the assignment of 120 long range NOEs and pseudo-NOEs (to simulate hydrogen bonds). The model, manual NOEs, and the unassigned peak lists were analyzed by CYANA (43), which calculated more structures and provided 1761 additional NOE assignments. For further refinement, the program GENMR utilized the chemical shifts and the NOE assignments (44). The 20 lowest energy structures were submitted to the Protein Data Bank, accession code 2MC9. Table 2 shows selected NMR statistics of the structure (45, 46). Fig. 7a shows a topological diagram of the Cat r 1 cyclophilin. The NMR structure revealed a typical Cyp fold consisting of a β-barrel of seven antiparallel β-strands and two surrounding α-helices. A search for related structures in the

**FIGURE 4.** Peptidyl-prolyl cis-trans-isomerase assay. Cat r 1 (30 nM) catalyzes isomerization of the substrate N-succinyl Ala-Ala-Pro-Phe p-nitroanilide (100 μM) and thus exhibits a functional activity typical for Cyps. Curve a, time course of the Cat r 1-catalyzed isomerization; curve b, thermal isomerization in the absence of Cat r 1 (control).

**FIGURE 5.** Cross-reactivity between Cyps. a, to evaluate cross-reactivity between Cat r 1 and fungal Cyps, pooled serum from five Cat r 1-allergic subjects was preincubated with increasing amounts of either Asp f 11 or Mala s 6 and was added to Cat r 1-coated ELISA plates. Cat r 1 and BSA were used as positive and negative controls, respectively. Binding of serum IgE to plate-bound Cat r 1 was inhibited by fungal Cyps in the fluid phase, indicating their cross-reactivity to Cat r 1. b, rabbit antibodies against plant and animal Cyps recognized Cat r 1 and its loop deletion mutant Cat r 1 in immunoblots. SDS-PAGE lanes are as follows. Lane 1, marker; lane 2, Cat r 1; lane 3, ΔCat r 1. Immunoblot lanes are as follows. Cat r 1 (lanes 1 and 3) and ΔCat r 1 (lanes 2 and 4) were detected with rabbit antibody against plant (lanes 1 and 2) or animal (lanes 3 and 4) Cyp. IgE-specific immunoblotting showed that serum IgE from two Catharanthus-sensitized patients (patient 1 (lanes 7 and 10) and patient 2 (lanes 8 and 11)) but not the non-atopic control (lanes 9 and 12) recognized both cat r 1 (lanes 7–9) and its loop deletion mutant (lanes 10–12).
Protein Data Bank revealed that the overall fold is most closely related to the human (1NMK) and the Plasmodium (1QNH) Cyp structures (47–49). The biggest difference from the human structure is the presence of additional residues that are found in the plant and parasite species after the first helix (Fig. 7b, a–f). In the NMR structure of Cat r 1, this loop is slightly more extended away from the center of mass compared with the structures from Leishmania and Caenorhabditis elegans (Fig. 7b, a–c). Compared with the human and fungal Cyp structures, this loop appears to make a large protuberance on the structure (Fig. 8, a–c). The structure of the human Cyp also contains a peptide ligand in the active site. Fig. 8, a and d, compares the electrostatic surface of Cat r 1 with the human Cyp displayed in the same orientation with the substrate peptide. The active site cleft appears slightly broader in Cat r 1 and has a more acidic character, indicating that Cat r 1 may have an optimal activity for peptides with different residues flanking the isomerizing proline than human Cyp.

### Structural Similarities and Differences between Prokaryotic, Plant, Fungal, and Animal Cyps

Fig. 9 shows the multiple-sequence alignment of Cat r 1 with Cyps from bacterial, fungal, plant, nematode, arthropod, and mammalian sources. It shows the presence of the RSGKPLH loop (red arrow) predominantly in plant Cyp sequences (with the exception of a nematode Cyp) with the regions of highest identity lying between amino acids 60 and 139 of the Cat r 1 sequence. A shallow pocket region on the barrel surface has been shown as the PPlase active site with an Arg residue (R in Fig. 9; red underlined) identified as a crucial catalytic residue in Cyps. Cyclosporin A also binds to the same area, making interactions with Cyp residues, including the same Arg (50, 51).

Two highly conserved, solvent-exposed stretches of residues (106SMANAGPNTNGSQFFI121 and 127WLDGKVFG136; Cat r 1 residue numbers; regions R1 and R2 in Fig. 9) were previously identified in the Cyp allergen sequences and believed to be potential candidates for cross-reactivity (52). In Cat r 1, they exist as closely located solvent-exposed patches and together contribute 11011 Å² of side chain-accessible surface area (i.e. more than 13%) to the total solvent-accessible antigenic surface (~8551 Å²) of the Cat r 1 molecule. They may be responsible for cross-reactivity among Cyps.

In order to address cross-reactivity with a more global bioinformatic analysis, Fig. 10a presents a dendrogram showing the relationships between Cyps from various species groups. The sequence identities among the members of these groups are all very high, typically greater than 60% (data not shown). Because common epitopes will usually contain common residues, we prepared three comparisons of the surface residue conservation among plant, fungi, and mammalian Cyp in Fig. 10b using the program ConSurf (37). The comparison performed a multisequence alignment, grouping plants with fungi (Fig. 10b, top panels), grouping mammals with plants (Fig. 10b, middle panels), and grouping...
fungi with mammals (Fig. 10b, bottom panels). The degree of sequence identity at each site is then color-coded on the structures. In all three permutations, the highest degree of homology is found adjacent to the active site (left column), and lower homology is found on the reverse side (right column). The high degree of surface conservation for a large contiguous surface area suggests a strong possibility of cross-reactivity among all groups, consistent with the present data. The analysis also predicts that cross-reactivity is more likely due to recognition of residues surrounding the active site.

In an attempt to interpret and quantify the structural comparisons, we calculated the percentage of side chain-accessible surface area with a ConSurf conservation score greater than 4. A high percentage would indicate a high likelihood of cross-reactivity, and vice versa. This percentage of surface conservation for the three permutations is as follows: fungi/plants, 50%; mammals/plants, 61%; fungi/mammals, 50%. Because fungal
and mammalian Cyp allergens are known to cross-react, it follows that the higher percentage predicts that the other groups should cross-react as well.

**DISCUSSION**

Cyps have been the subject of considerable scientific interest due to their high biochemical and clinical relevance. Proteins of this family have been isolated from bacteria, fungi, protozoa, nematodes, and several groups of animals, including protozoans, nematodes, arthropods, birds, and mammals, indicating their universally significant biological function. Cyps are encoded by 6–10-member gene families and are highly conserved throughout evolution. Three Cyp genes and at least seven pseudogenes were identified from the human genome (53).

Arabidopsis Cyp was cloned more than 30 years ago, which was followed by cloning of CypA genes from numerous plant species, such as tomato, rice, and maize (31). However, prior to this study, no plant Cyp allergen structure has been reported, with no data or limited structural data on plant-derived Cyp proteins in general.

Cyps (pfam ID: PF00160) have recently been designated as an emerging family of cross-reactive pan-allergens (9). Due to their high sequence similarity and demonstrated cross-reactivity with human Cyp, it has been suggested that the human Cyp might act as a relevant autoallergen in Cyp-sensitized individuals (9). In fact, 50% of patients with allergic broncho-pulmonary aspergillosis and 70% of patients with atopic eczema show a positive intradermal test reaction with Aspergillus Cyps (Asp f 11 and Asp f 27), indicating the presence of functional anti-Cyp serum IgE in these patients. Similarly, 30% of the aller-
gic broncho-pulmonary aspergillosis and atopic eczema patients show sensitization to *Malassezia* Cyp Mala s 6. In fact, both *in vitro* immunoassays (immunoblots and ELISA) and *in vivo* intradermal tests show the relevance of Cyp in allergic broncho-pulmonary aspergillosis and atopic eczema, two chronically relapsing inflammatory diseases (54, 55). Thus, structural and immunological properties of fungal Cyp allergens have been the focus of several investigations. However, there are limited data linking structure to immunological properties in the case of plant-derived Cyps.

Here we describe the identification of Cat r 1, an 18-kDa protein from *C. roseus* pollen, as a relevant allergen, which is homologous to Bet v 7 in sequence. Following SDS-PAGE and blotting with potential allergic sera, a common 18 kDa band was sequenced, and the allergen was cloned by screening a *C. roseus* cDNA library. Database analysis and functional characterization revealed that Cat r 1 is a member of the Cyp family. A prior periodate treatment of blotted *C. roseus* pollen caused a considerable decrease of IgE reactivity to several detected bands except for the 18 kDa Cat r 1 band (data not shown). This suggested that, in contrast to the other bands, glycans are not involved in IgE binding to Cat r 1. The sequence of Cat r 1 has two potential N-glycosylation sites, N<sup>78</sup>GT and N<sup>115</sup>GS (shown in boldface type in Fig. 1), thus signifying that Cat r 1 could be a glycoprotein. However, in the structure, one asparagine residue (Asn<sup>115</sup>) is buried within the protein structure, whereas the other one (Asn<sup>78</sup>) is only partially exposed, and the molecular mass calculated from the sequence of Cat r 1 (18.3 kDa) is very close to that determined by SDS-PAGE (18.7 kDa). Moreover, because deglycosylation of *C. roseus* extract by periodate treatment had no effect on Cat r 1 IgE-binding, and unglycosylated recombinant Cat r 1 was detected by allergic patients' IgE equivalently to natural Cat r 1, we think that glycans are not involved in the IgE binding to Cat r 1.

Recent analysis of allergenic proteins demonstrates that allergens are distributed into only a few protein families (56, 57). In fact, pollen allergens populate only 29 of more than 7000 protein families (57). Of these, profilin constitutes a conserved homologous group with high sequence identities (70–85%) while showing low identities (30–40%) with non-allergenic profilins from other eukaryotes, including humans. About 10 of the 29 pollen allergen families are also present in plant-derived foods (58). Like the profilins, Cyps represent a family of cross-reactive allergens. The solution structure of Cat r 1 showed overall similarity to Asp f 11, Mala s 6, and human CypA. Significant evolutionary conservation of solvent-exposed residues has been observed between plant and fungal Cyps, and ELISA inhibition experiments indicated a partial cross-reactivity.

![Dendrogram showing the evolutionary relationship between Cyp proteins from bacteria, protozoa, fungi, plant, and animal sources. Uniprot accession numbers have been shown.](image-url)

**FIGURE 10.** A, dendrogram showing the evolutionary relationship between Cyp proteins from bacteria, protozoa, fungi, plant, and animal sources. Uniprot accession numbers have been shown. B, sequence conservation mapped on the surface of Cat r 1 based on a multisequence alignment grouping plants with fungi, grouping mammals with plants, and grouping fungi with mammals. The highest degree of conservation was found adjacent to the active site. The sequences diverged through evolution but show considerably conserved antigenic surface.
among these Cyps. Thus, our study does not support the observation that plant Cyps (described for Bet v 7) do not cross-react (12). This could be due to differences in dominant cross-reactive epitopes recognized by the patients. In fact, the cross-reactivity is likely to be more significant than previously discussed. This notion is supported by the fact that the putative antigenic/allergenic surface area near the active site is strongly conserved between species. Deletion of the plant-specific loop did not significantly reduce rabbit antibody binding to Cat r 1, indicating the retention of the epitopes recognized by the polyclonal antibody. 

Taken together, we present a detailed molecular characterization of the allergen Cat r 1, which shows more than 60% sequence identity with fungal allergens Asp f 11 and Mala s 6. This is the first report on the solution structure of a plant Cyp allergen with detailed analysis of its antigenic surface and cross-reactivity. The data obtained here should be helpful for structure-based epitope mapping, molecular diagnostics, and component-resolved immunotherapy of Cyp allergic patients.

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