

Short communication

Isolation of cypress gibberellin-regulated protein: Analysis of its structural features and IgE binding competition with homologous allergens

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ABSTRACT

The presence in cypress pollen of an important allergen, belonging to the gibberellin-regulated protein (GRP) family, has been suggested for many years. However, it has never been isolated and sometimes the homologous peach allergen, Pru p 7, has been used as a surrogate to perform immunological investigations. The aim of this study has been the isolation and molecular characterization of the GRP contained in the *Cupressus sempervirens* pollen. This protein, named Cypmaclein, has been purified from the natural source using conventional biochemical methods consisting in different chromatographic separations. Cypmaclein has been identified by direct protein sequencing of the N-terminal region and of internal fragments of the molecule. In SDS-PAGE, its apparent molecular mass is slightly higher than that of Pru p 7. Nevertheless, the mass spectrometry experiments reveal that the exact molecular mass of Cypmaclein (6821.88 Da) is very close to that of Pru p 7 (6909.90 Da). Two regions of Cypmaclein have been sequenced providing 50% of its primary structure. A high overall sequence identity of Cypmaclein with all the analyzed GRP has been observed, although in the N-terminal region the high identity is limited to the homolog of *Cryptomeria japonica*. In circular dichroism experiments Cypmaclein produced a spectrum overlapping that of Pru p 7. However, the comparative analysis of Cypmaclein, Pru p 7 and Pun g 7 IgE reactivity revealed a behavior that was not completely overlapping, thus suggesting that the IgE epitopes are only partially shared. In single point highest inhibition achievable assays performed with the FABER test, Cypmaclein efficiently competed with the allergenic peach and pomegranate GRP in the binding of specific IgE of patients sensitized to Pru p 7. In conclusion, the natural cypress pollen GRP has been isolated for the first time, its structural features have been investigated and its cross-reactivity with Pru p 7 and Pun g 7 has been demonstrated. This protein is now available for further investigations aimed at understanding its clinical relevance in the allergy to cypress pollen. In addition, the prevalence of sensitization directly to Cypmaclein, and not limited to the homologs, can be defined.

1. Introduction

Gibberellin-regulated proteins (GRP), also known as Snakin/GASA peptides (Nahirñak et al., 2019), are ubiquitous proteins in mono- and dicotyledonous plants. The expression of GRP is up-regulated by gibberellins (GA), which are a class of natural phytohormones consisting in tetracyclic diterpene acids produced by plants and some fungi and bacteria (Aubert et al., 1998). GA and GRP play a crucial role in plant growth and developmental processes, including germination, cell division, dormancy, flowering and fruit development (Muhammad et al., 2019). Therefore, the concentration of GRP in plants is finely regulated

as a function of the specific tissue and developmental stage. In addition to the normal physiological processes, the GA-induced GRP level is further affected in response to abiotic stresses, such as salt, drought, cold and heat stress (Muhammad et al., 2019). The external application of synthetic GA, generally by spraying, is a widespread agricultural treatment used to increase and improve crop production (Hedden and Sponsel, 2015; Li-jun et al., 2008). Therefore, GA treatments are also applied to a variety of foods for human uses where they affect the amount of produced GRP.

GRP are small proteins the function of which in plants is still unclear. They are synthesized as precursors with a higher molecular mass

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containing a signal peptide that is removed in the mature form of about 7 kDa. In the mature form two regions can be detected, a short N-terminal stretch with low sequence conservation and a highly conserved C-terminal region containing the 12-cysteine pattern characteristic of this protein family (Muhammad et al., 2019).

The GRP isolated from peach fruit, named peamaclein, is the first GRP to be identified as an allergen and was named Pru p 7 (Tuppo et al., 2013, 2014). Later, allergenic homologs found in other fruits were described, namely Pru m 7 from Japanese apricot (Inomata et al., 2017), Pun g 7 from pomegranate (Tuppo et al., 2017) and Cit s 7 from orange (Inomata et al., 2018). A high variability of the protein concentration in the natural source was reported for Pru p 7 and Pun g 7, that were sometimes found in high amounts and in other cases proved to be even undetectable in some fruit cultivars or in different batches of the same cultivar (Tuppo et al., 2017, 2013). Therefore, at least in peach and pomegranate, the GRP concentration is not constant and the possibility that sometimes these proteins might even be absent cannot be excluded. The factors affecting the GRP content in these fruits have not been investigated yet. Therefore, it is not known whether the endogenous production or external spraying of GA, or the combination of both, is the main cause of the occasional GRP increase in these foods.

The presence of an allergenic GRP in cypress pollen has been suggested following IgE immunoblot inhibition using sera of patients allergic to cypress pollen. In particular, an IgE cross-reactivity was observed with two GRP, the potato snakin-1 (Sénéchal et al., 2018) and the recombinant Pru p 7 (Klingebiel et al., 2019). The cypress molecule that was proposed to cross-react with GRP is a protein named BP-14 on the basis of its migration in SDS-PAGE with an apparent molecular mass of 14 kDa (Shahali et al., 2012). This molecular mass was in contrast with that of Pru p 7 (about 7 kDa). BP-14 was analyzed by mass spectrometry after in-gel digestion that allowed the identification of a fragment with a structural similarity with Pru p 7 (Sénéchal et al., 2018). Nonetheless, the identity of the GRP cross-reacting molecule contained in the cypress pollen has not been published yet.

In this context, the aim of this study has been the isolation and molecular characterization of the GRP protein contained in the *Cupressus sempervirens* pollen. In addition, the properties of the isolated protein have been compared with those of homologous GRP, such as the peach allergen Pru p 7.

2. Materials and methods

2.1. Purification of Cypmaclein from cypress pollen

Cypress (*C. sempervirens*) pollen was purchased from IBERPOLEN SL (Jaén, Spain). The pollen proteins were extracted overnight, at 4 °C, in a buffer containing 50 mM NaP pH 7.4, 1 M NaCl, 5 mM EDTA and 0.05 mg/ml PMSF. Next, the sample was centrifuged at 18,800 x g for 45 min and the supernatant, representing the protein extract, was collected. The extract was then dialyzed against 10 mM Tris–HCl, pH 7.2 and loaded on a DE52 (Whatman, Brentford, UK) column, equilibrated in the same buffer. Cypmaclein was eluted in the column flow-through that was loaded on a SP-Sepharose column (Amersham Biosciences, Uppsala, Sweden), equilibrated in 10 mM Tris–HCl, pH 7.2 (buffer A). The elution was carried out by increasing the concentration of buffer B (Tris–HCl 10 mM, pH 7.2, containing 0.5 M NaCl). The fractions containing low molecular weight proteins were detected by SDS-PAGE and pooled. Further purification was achieved by RP-HPLC on a Vydac (Deerfield, IL, USA) C8 column (4.6 x 250 mm), using a Beckman System Gold apparatus (Fullerton, CA, USA). Elution was performed by a multistep linear gradient of eluent B (0.08% TFA in acetonitrile) in eluent A (0.1% TFA) at a flow rate of 1 ml/min. The eluate was monitored at 220 and 280 nm.

2.2. Estimation of the protein concentration

The protein concentration in the extract was estimated by BIO-RAD Protein Assay (Bio-Rad, Milan, Italy), using calibration curves made with bovine serum albumin.

The estimation of the purified protein concentration was obtained on the basis of the molar extinction coefficient at 280 nm ($5220 \text{ M}^{-1} \text{ cm}^{-1}$) of the homologous protein of *Cryptomeria japonica*, coded by the clone BY902962, found in the EST database. This coefficient was calculated using the ProtParam algorithm on the ExPASy Bioinformatics Resource Portal (www.expasy.org).

2.3. Mass spectrometry experiments

The molecular mass of the purified protein was determined on a QSTAR® Elite System equipped with a Nano-electroSpray® Source (Applied Biosystems, San Diego, CA, USA). The analysis was performed by loading 1 µl of Cypmaclein at the concentration of 10 pmol/µl. Spectral deconvolution and mass reconstruction were performed using the algorithm “Mass Reconstruction” in the Analyst QS software.

2.4. Amino acid sequencing

The purified protein was denatured and its sulphhydryl groups were carboxymethylated as already described (Di Donato et al., 1986). 700 pmol of the denatured and carboxymethylated protein were subjected to automated direct sequencing of the N-terminal region with a Protein Sequencer PPSQ–33B (Shimadzu Corporation, Tokyo, Japan).

In addition, 3 nmoles of denatured and alkylated protein were subjected to proteolytic cleavage by trypsin (Roche Diagnostics, Mannheim, Germany), following the manufacturer's instructions. The recovered peptides were separated by RP-HPLC and some of them were analyzed by direct sequencing.

2.5. Circular dichroism (CD) experiments

CD spectra were recorded on a JASCO J-810 spectropolarimeter (Easton, MD, USA) as already reported (Tuppo et al., 2013). A quartz cell of 0.1 cm path length was used to record the spectra over the wavelength range 250–190 nm with a bandwidth of 1.0 nm. Measurements were performed in 5 mM sodium phosphate pH 7.4, at 25 °C, using a protein concentration of 0.1 mg/ml. Each spectrum was baseline corrected for the contribution of the solvent.

2.6. Specific IgE detection and IgE inhibition experiments by the FABER® multiplex testing system

The FABER® test (ADL) is a multiplex in vitro serological test allowing the detection of specific IgE antibodies from allergic subjects (Alessandri et al., 2017; Giangrieco et al., 2019; Tuppo et al., 2018). The FABER version used to perform this study (FABER 244-122-122) bears 244 allergenic preparations, representing 122 purified molecular allergens and 122 allergenic extracts, all conjugated to nanobeads. Among the regular allergenic molecules two GRP proteins are available, namely Pru p 7 and Pun g 7.

The direct IgE detection was obtained from a set of biochips also including Cypmaclein and the cypress extract, Cup s, spotted for experimental purposes. Before the immobilization on the FABER biochip, these samples were coupled to nanobeads following the same procedure applied to all the other allergenic preparations. This multiplex diagnostic test allowed the detection of specific IgE to each of the allergenic preparations contained in the regular FABER biochip, also including Cypmaclein and Cup s in a single run.

To obtain information on allergen epitopes shared by homologous proteins, the SPHIAa assay (Palazzo et al., 2013; Pasquariello et al., 2012) was used with some modifications.

Table 1
Features of the sera used in IgE-inhibition experiments.

PATIENT DETAILS			SYMPTOMS		FABER 244 (FIU) [*]	
Number	Gender	Age	Respiratory	Food	Pru p 7	Pun g 7
1	F	35	R, C, BA	OAS	12.94	16.62
2	F	27	R	OAS, ANG, U, G	31.26	0
3	F	8	R	OAS, ECZ	7.42	8.05
4	F	15	R, C, BA	OAS	28.45	4.42
5	M	16	R, C, BA	OAS	5.04	10.80

ANG, angioedema; BA, bronchial asthma; C, conjunctivitis; ECZ, eczema, G, gastritis; OAS, oral allergic syndrome; R, rhinitis; U, urticaria.

* FABER International Units; positive value FIU ≥ 0.01 .

Briefly, 0.12 ml of a single undiluted serum were co-incubated with 0.12 ml of a solution containing 0.1 mg of the purified allergen or the extract preparation. Next, the IgE binding inhibition was evaluated by running the FABER test and recording the residual IgE binding on the allergens spotted on the biochip. Reference values for the lack of IgE binding inhibition were obtained for each serum by running control samples where the allergen solution was replaced with buffer only. The inhibition values were calculated in real time by a specific procedure developed within the InterAll software (version 5.0, ADL).

2.7. Patients' sera

Thirty-one characterized sera, being IgE positive for either Pru p 7 or Pun g 7 on the regular FABER test, were selected and used to define IgE co-reactivity of the three GRP allergens (Pru p 7, Pun g 7 and Cypmaclein) spotted on the FABER biochip.

To perform the Single Point Highest Inhibition Achievable assay (SPHIAa), five characterized sera (numbered 1–5) were selected from the InterAll data bank (version 5.0, ADL) because they had been shown to contain IgE specifically binding the peach peamaclein, Pru p 7, following the FABER test (Table 1). Four of them proved also to be IgE positive to the pomegranate homologous protein, Pun g 7. One serum (number 2) was not IgE positive to Pun g 7. In addition, the selected sera were IgE positive to the *C. japonica* pollen extract (Cry j) and to the genuine marker of cypress pollen sensitization, Cup a 1.

All patients gave their informed consent to the use of their clinical data for research purposes in an anonymous form. In view of the purely comparative nature of this study, along with the fact that all venous blood samplings were part of routine clinical practice and that a residual part of the routine sample was used for inhibition experiments, a formal approval by the Ethical Committee was not necessary.

2.8. Statistics and graphics

1way ANOVA distribution test was used to compare IgE distribution of the three datasets.

Spearman r test was used to evaluate the correlation of IgE values between two dataset.

Upgraded Prism 5 software (Graphpad Software Inc., San Diego, CA, USA) was used for statistical evaluation and for producing graphs.

3. Results

3.1. Purification of Cypmaclein from *C. sempervirens* pollen

The protein profile of the extract (0.64 mg/ml) obtained from *C. sempervirens* pollen is shown in Fig. 1A. The extract was subjected to anion exchange chromatography and the proteins contained in the flow-through were next separated by a cation exchange resin (Fig. 2A). The fractions 36–41 (Fig. 2A) were pooled and further separated by RP-HPLC. The elution profile showed a main peak eluted at 22 min

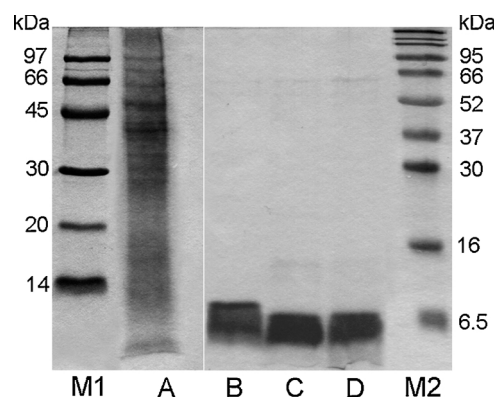


Fig. 1. Reducing 15% SDS-PAGE analysis. Following electrophoresis, proteins were stained with Coomassie Brilliant Blue. M1 (GE-healthcare, Uppsala, Sweden) and M2 (Euroclone, Pero, Milano), molecular weight markers; (A) *C. sempervirens* extract (60 μ g); (B) Cypmaclein (4 μ g); (C) Pru p 7 (4 μ g); (D) Pun g 7 (4 μ g).

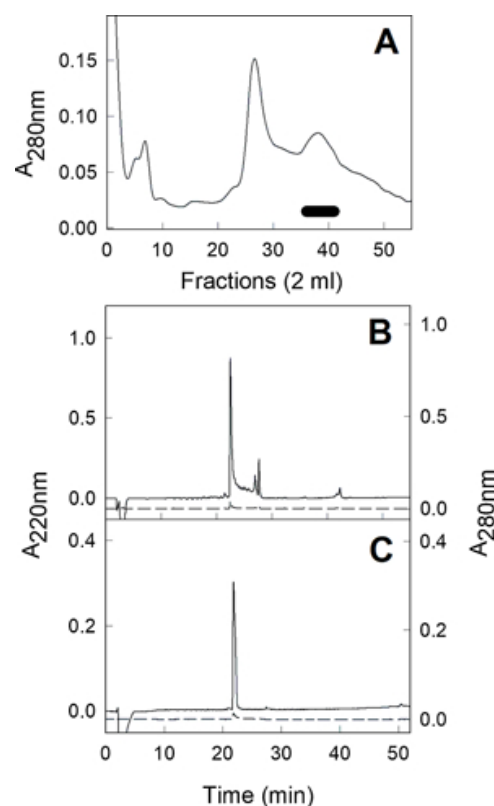


Fig. 2. Purification of Cypmaclein by chromatographic separations. (A) separation by cation exchange chromatography of the cypress protein fraction recovered in the flow-through of the anion exchange chromatography. Black bar indicates the fractions 36–41 containing small proteins. (B) Separation by RP-HPLC of the pooled fractions 36–41 obtained by cation exchange chromatography. (C) RP-HPLC of the purified Cypmaclein.

(Fig. 2B). This component was collected, denatured, carboxymethylated and then subjected to automated N-terminal amino acid sequencing. Sixteen cycles of Edman degradation produced the following amino acid sequence: AQIDCDKECNRRCSKA.

Using this procedure, 1 mg of the protein component was obtained from 70 g of pollen. The pure protein showed a single band on SDS-PAGE (Fig. 1B), a single peak on RP-HPLC (Fig. 2C) and a single N-terminal amino acid sequence.

This cypress protein was named by us as Cypmaclein.

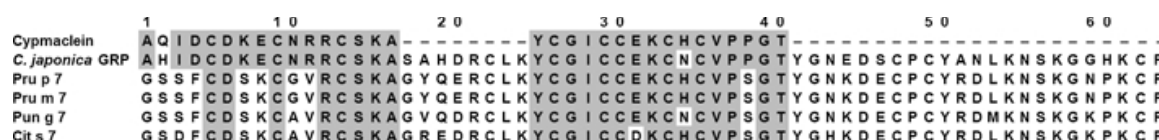


Fig. 3. Multiple sequence alignment of GRP. The sequenced fragments of Cypmaclein are aligned with GRP from *C. japonica* (EST clone [BY902962](#)), Pru p 7 from peach (Uniprot [P86888](#)), Pru m 7 from Japanese apricot (GenBank Protein [XP_016648029.1](#)), Pun g 7 from pomegranate (Uniprot [C0HKC0](#)) and Cit s 7 from orange (Uniprot [A0A067D4T6](#)).

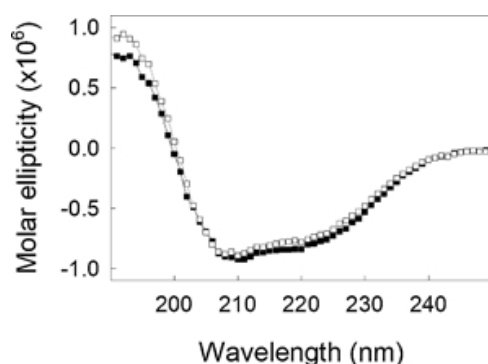


Fig. 4. CD spectra of Cypmaclein (filled squares) and Pru p 7 (hollow squares).

3.2. Identification of Cypmaclein as a GRP protein of cypress pollen

A similarity search performed in UniProtKB (www.expasy.org) with the BLASTP algorithm on the ExPASy server, using the N-terminal sequence (AQIDCDKECNRRCSKA) of the protein isolated from cypress pollen, produced unclear results. However, a further similarity search carried out using the same N-terminal sequence to query the Expressed Sequence Tags (EST) database by the BLAST algorithm at NCBI (www.ncbi.nlm.nih.gov/Blast.cgi) detected a cDNA clone, BY902962, deriving from male strobili of *C. japonica*. This clone coded for a protein showing a sequence similarity with GRP proteins (Fig. 3), including the peach allergen Pru p 7.

3.3. Primary structure analysis

Fig. 3 highlights a high conservation of the primary structure in all the aligned GRP. However, the sequence identity decreases in the N-terminal region. In particular, it can be observed that the first eleven residues of Cypmaclein are more highly conserved in the corresponding region of the protein from *C. japonica* rather than in the GRP of the three fruits. An additional confirmation that the protein isolated from *C. sempervirens* pollen is a homolog of peach peamaclein was obtained by sequencing the N-terminal region of an internal fragment produced by trypsin digestion of the denatured and carboxymethylated molecule. The obtained amino acid sequence was the following: YCGICCEKCHCVPVPGT. Clearly this fragment was generated by a missing cleavage at the lysine (K) because of the presence of an adjacent acidic residue (E). This peptide was unambiguously aligned with the residues 26–41, located in a highly conserved region of GRP (Fig. 3). In fact, 14 or 15 of the 16 residues of the peptide were shared with each of the aligned GRP. Assuming that this protein comprises 63 amino acids, like the homologous molecules, the available amino acid sequence of 32 residues represents more than 50% of the Cypmaclein primary structure.

The identity between the available sequence of Cypmaclein displays 94% of identical residues with the corresponding regions of *C. japonica* GRP. The comparison with peach, pomegranate and orange GRP reveals a similarly high sequence identity in the region comprising the residues 26–41. In contrast, the residues of Cypmaclein conserved in the fruit GRP decrease to 50% when the first 16 residues of the N-terminal region are compared. The sequence identities of Pru p 7 with Pru m 7, Pun g 7, Cit s 7 and Cry j GRP are 100%, 90%, 87% and 68%,

respectively.

3.4. Cypmaclein molecular mass

Cypmaclein migrated in reducing SDS-PAGE with an apparent molecular mass slightly higher than that observed for Pru p 7 (Fig. 1). The average apparent molecular mass values, calculated using two different molecular markers as references (Fig. 1, Lanes M1 and M2), were 9.5 kDa, 8.5 kDa and 8.5 kDa for Cypmaclein, Pru p 7 and Pun g 7, respectively. (Fig. 1).

The exact value of the molecular mass of Cypmaclein, obtained by the mass spectrometry experiments, was 6821.88 Da.

3.5. Structural analysis by circular dichroism experiments

Fig. 4 shows that far-UV CD spectrum recorded at 25 °C for the Cypmaclein is overlapping that of the homologous molecule from peach, Pru p 7. This result indicates that, like Pru p 7, Cypmaclein displays a mixed alpha-helical/beta-sheet structure. In fact, the curve is characterized by the two minima typical of the alpha-helix, around 202 and 206 nm, although the non-fully canonical shape reflects the contribution of other secondary structures, such as beta structures and/or random coils.

3.6. Direct IgE binding

The direct evidence of the IgE binding by Cypmaclein was obtained using the FABER test. Sixteen of 31 samples showing IgE binding to Pru p 7 and/or Pun g 7 were scored also positive to Cypmaclein (Fig. 5, Panel A). The statistical evaluation applied to the three IgE dataset, as shown in Fig. 5, Panel A, showed a significant difference of the IgE value distributions. IgE correlations of the three analyzed GRP are shown in Fig. 5, panels B, C and D. Statistical evaluation by Spearman r test showed a significant though not very high correlation between IgE values of Pru p 7/Pun g 7 and Pru p 7/Cypmaclein, whereas the correlation between Pun g 7/Cypmaclein was found not significant.

3.7. IgE inhibition experiments

The SPHIAa on FABER was used to run specific IgE inhibition experiments using the *C. sempervirens* pollen extract (Cup s), the purified Cypmaclein and Pru p 7. The five sera used for these experiments were from patients sensitized to Pru p 7, *C. japonica* pollen and Cup a 1. Fig. 6 shows that the cypress extract completely inhibits the IgE binding to Pru p 7. In fact, the pre-incubation of the extract with each one of the five selected sera abolished the IgE binding to the peach allergen spotted on the FABER biochip. A complete IgE binding inhibition to Pun g 7 was also observed, except for serum 2. In fact, this patient, although IgE positive to Pru p 7, did not have IgE recognizing pomegranate GRP. As expected, the cypress extract also caused a high inhibition of IgE binding to the *C. japonica* extract and to Cup a 1.

Cypmaclein and Pru p 7 completely inhibited the IgE binding to Pru p 7 and Pun g 7 spotted on the FABER biochip (Fig. 6). Inhibition of IgE binding to Pun g 7 was not detected for serum 2 because it was devoid of specific IgE recognizing pomegranate GRP. Similar to Pru p 7, Cypmaclein did not inhibit Cup a 1, thus excluding the possibility that the

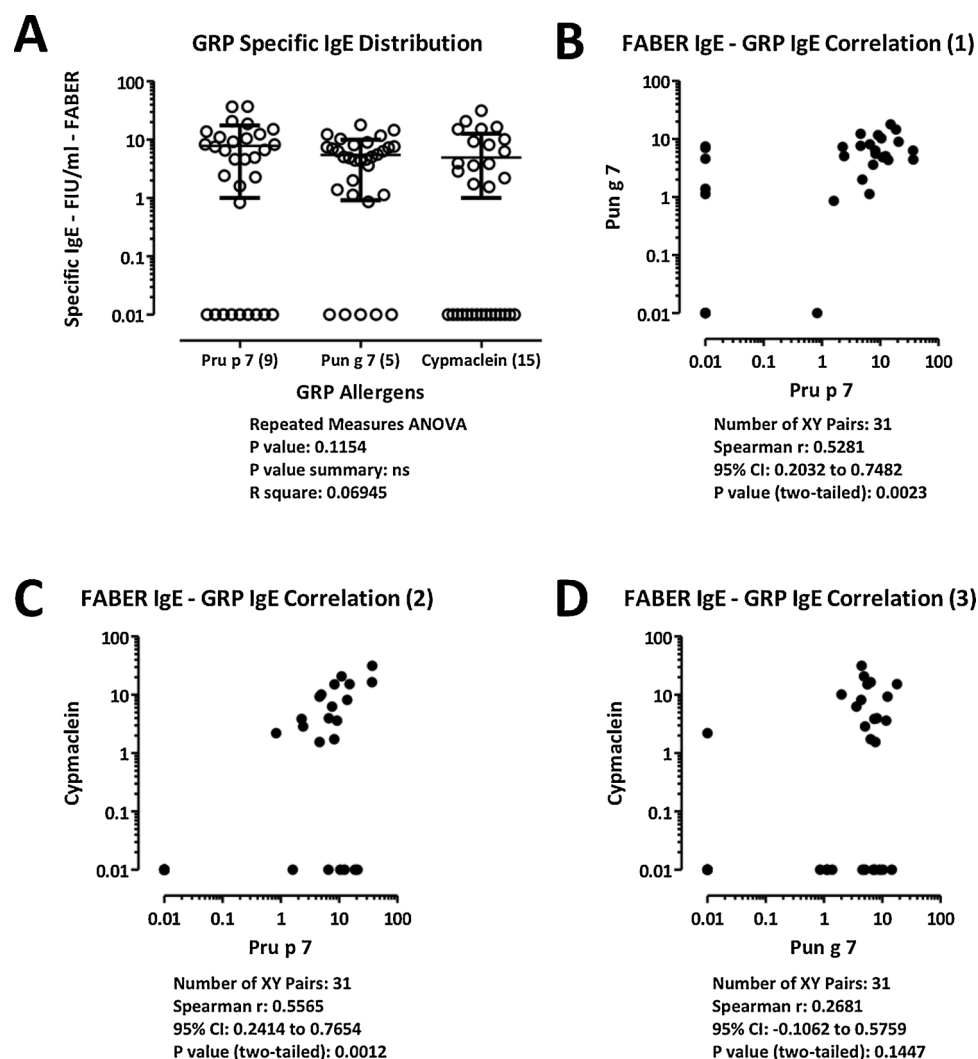


Fig. 5. IgE binding of Cypmaclein, Pru p 7 and Pun g 7 spotted on the FABER biochip. Panel A: comparative IgE distribution obtained with 31 sera tested IgE positive to GRP allergens. Values in the brackets of column labels report the number of negative patient per each allergen. Panels B, C, and D: IgE value correlations for paired GRP. Statistics are reported below each graph.

cypress GRP preparation might contain Cup a 1-like proteins from *C. sempervirens* (Cup s 1) as a contaminant. In addition, the cypress and peach GRP did not cause a significant IgE inhibition on the *C. japonica* pollen extract. This result suggests that, in the used sera, the fraction of IgE recognizing the GRP is smaller compared to that including all the IgE specific for the other allergens contained in the *C. japonica* extract. Before drawing any conclusion investigations on *C. japonica* pollen proteins should be carried out focusing the presence of GRP proteins.

4. Discussion

This study describes the isolation of an IgE-binding GRP from the pollen of *C. sempervirens*, that has been named Cypmaclein. The procedure used was based on successive chromatographic separations. The protein behavior during the ion exchange chromatographies indicates that, like peach peamaclein, Cypmaclein is a basic molecule. The characterization of this protein demonstrates that, in contrast with the literature reports (Charpin et al., 2019), the molecular mass of the cypress GRP is 6821.88 Da, which is a value very similar to that reported (Tuppo et al., 2013) for Pru p 7 (6909.90 Da). However, in SDS-PAGE the behavior of Cypmaclein is slightly different compared to that of the homologous molecules from peach, Pru p 7, and pomegranate, Pun g 7. In fact, Cypmaclein shows an apparent molecular mass in SDS-PAGE of

about 9.5 kDa, whereas Pru p 7 and Pun g 7 migrate at a slightly lower molecular mass of about 8.5 kDa. A migration in SDS-PAGE not completely consistent with the true molecular mass of a protein is not surprising and it is well documented in the literature. Compared to the true values, in SDS-PAGE proteins can show an apparent molecular mass slightly higher, such as Act d 2 (Gavrović-Jankulović et al., 2002; Palazzo et al., 2013) and Act d 5 (Tamburrini et al., 2005), but even strongly different, such as the latex allergen Hev b 5 and its homologs (Giangrieco et al., 2019). Concerning GRP, it is not clear which is the cause of the slightly higher apparent molecular mass in our SDS-PAGE of Cypmaclein compared to Pru p 7 and Pun g 7. Nevertheless, this different behavior suggests structural differences between the cypress GRP and the two fruit homologs.

On the basis of the literature reports combined with the results of this study, it is conceivable that the cypress protein that was named BP-14 (Shahali et al., 2012) and proposed to cross-react with GRP (Klingebiel et al., 2019; Sénéchal et al., 2018) is the Cypmaclein described in this paper. BP-14 derives its name from its migration in SDS-PAGE with an apparent molecular mass of 14 kDa. We cannot exclude that the experimental conditions, for instance the concentration of reducing agents (Tamburrini et al., 2005) and the use of gradient or linear gels chosen to run the electrophoretic separations in different laboratories, could have affected the cypress GRP migration in SDS-PAGE,

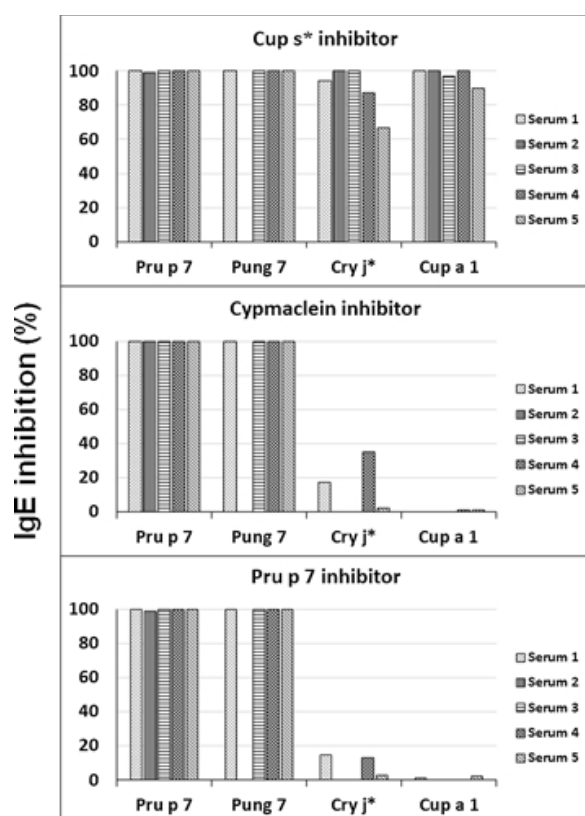


Fig. 6. IgE Inhibition assays using the FABER testing system with the SPHIAa method. Five individual sera of patients sensitized to Pru p 7 are used as providers of specific IgE. The analyzed antigens spotted on the biochip are Pru p 7, Pun g 7, *C. japonica* pollen extract (Cry j*) and Cup a 1. Inhibitors of IgE binding were *C. sempervirens* pollen extract (Cup s*, panel A), Cypmaclein (panel B) and Pru p 7 (panel C).

thus apparently generating discrepant molecular mass values for this protein. Now, the availability of the pure protein has allowed the unraveling of this issue with the determination of the true molecular mass (6821.88 Da) of cypress GRP by mass spectrometry.

The comparative primary structure analysis of the GRP of peach, pomegranate, orange, *C. japonica* and the available fragments of *C. sempervirens* highlights a high sequence conservation between these molecules, although they belong to different plant taxonomic orders of the Magnoliopsida or Pinopsida classes. In fact, the sequence identities range from 67%, when the distantly related *C. japonica* GRP is compared with Cit s 7, to 100% when the peach Pru p 7 is compared with the Japanese apricot Pru m 7. A high identity, 90%, is observed when Pun g 7 is compared with Pru p 7, although these two proteins belong to different taxonomic orders. Most of the amino acid positions, including the pattern of 12 cysteines, are conserved in all the compared proteins. The residue variations are clustered in fixed sequence stretches. Among these, the N-terminal region is found to be highly conserved if the GRP from the two Cupressales (order Pinopsida), namely Cypmaclein and the homolog from *C. japonica*, are compared. In contrast, this region is highly variable if the Cupressales proteins are compared with the three GRP from peach, pomegranate and orange, belonging to the class Magnoliopsida, orders Rosales, Myrtales and Sapindales, respectively.

The similarity between the complete primary structure of Cypmaclein and the other allergenic GRP so far identified cannot be evaluated since its sequence is only partially available. Nevertheless, CD experiments have revealed that the secondary structure, consisting of mixed alpha-helical/beta-sheet structure, is conserved between Cypmaclein and the peach homolog Pru p 7.

This study also reports for the first time the results obtained with

purified cypress GRP competing with the homologous allergens for the binding of specific IgE. In fact, Cypmaclein causes a complete inhibition of IgE binding to Pru p 7 and Pun g 7 immobilized on the FABER biochip, thus indicating that these three GRP have common IgE epitopes. However, an analysis with the FABER test of a large population (10,393 patients) reveals that 0.6% and 0.5% of subjects are sensitized to Pru p 7 and Pun g 7, respectively (data not shown). Only a subset of patients are co-sensitized to both peach and pomegranate GRP, thus indicating that some IgE epitopes are not shared between these molecules, despite their high sequence identity. Therefore, it can reasonably be hypothesized that the epitope sharing between Pru p 7 and the homologs that are structurally less similar, such as the cypress GRP, is even lower. The reported preliminary analysis of the IgE co-reactivity of three GRP allergens goes in this direction. When unselected samples were used an IgE reactivity to the three, or even to a single one protein, was found. Further studies on IgE reactivity in larger cohorts will provide additional information on the immunological features of these proteins.

In conclusion, a GRP IgE binding protein has been isolated for the first time in cypress pollen. This molecule is now available for further studies required to investigate its clinical relevance and the prevalence of sensitization to this protein, compared to different GRP allergens. In line with the concept “one does not fit all”, already described for other allergens (Alessandri et al., 2017; Bernardi et al., 2011), the availability and use of individual allergens will contribute to the diagnosis even in those cases where the sensitization is limited to one, or a few, protein(s) of an allergen family. The clinical impact of single GRP protein sensitization requires also the testing of other allergens to which patients are exposed when ingesting/inhaling GRP proteins.

Note

The cypress Cypmaclein was submitted to the WHO/IUIS Allergen Nomenclature Sub-Committee to be considered for inclusion in the nomenclature with the name Cup s 7.

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Declaration of Competing Interest

CA and AM as ADL stakeholder, develop, manufacture and distribute the FABER test as a diagnostic service. MC and CR as employees of CAAM, LT and IG as employees of ADL, contribute to develop, manufacture and perform the FABER test. MAC and MT receive funding from ADL.

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References

- Alessandri, C., Ferrara, R., Bernardi, M.L., Zennaro, D., Tuppo, L., Giangrieco, I., Tamburrini, M., Mari, A., Ciardiello, M.A., 2017. Diagnosing allergic sensitizations in the third millennium: why clinicians should know allergen molecule structures. *Clin Transl Allergy* 7, 21.
- Aubert, D., Chevillard, M., Dorne, A.M., Arlaud, G., Herzog, M., 1998. Expression patterns of GASA genes in *Arabidopsis thaliana*: the GASA4 gene is up-regulated by gibberellins in meristematic regions. *Plant Mol Biol* 36, 871–883.
- Bernardi, M.L., Giangrieco, I., Camardella, L., Ferrara, R., Palazzo, P., Panico, M.R., Crescenzo, R., Carratore, V., Zennaro, D., Liso, M., Santoro, M., Zuzzi, S., Tamburrini, M., Ciardiello, M.A., Mari, A., 2011. Allergenic lipid transfer proteins from plant-

- derived foods do not immunologically and clinically behave homogeneously: the kiwifruit LTP as a model. *PLoS One* 6, e27856.
- Charpin, D., Pichot, C., Belmonte, J., Sutra, J.P., Zidkova, J., Chanez, P., Shahali, Y., Sénéchal, H., Poncet, P., 2019. Cypress pollinosis: from tree to clinic. *Clin. Rev. Allergy Immunol.* 56, 174–195.
- Di Donato, A., Galletti, P., D'Alessio, G., 1986. Selective deamidation and enzymatic methylation of seminal ribonuclease. *Biochemistry* 25, 8361–8368.
- Gavrović-Jankulović, M., Ćirković, T., Vucković, O., Atanasković-Marković, M., Petersen, A., Gojčić, G., Burazer, L., Jankov, R.M., 2002. Isolation and biochemical characterization of a thaumatin-like kiwi allergen. *J. Allergy Clin. Immunol.* 110, 805–810.
- Giangrieco, I., Ricciardi, T., Alessandri, C., Farina, L., Crescenzo, R., Tuppo, L., Ciancamerla, M., Rafaiani, C., Bernardi, M.L., Digilio, A.F., Cobucci-Ponzano, B., Tamburrini, M., Mari, A., Ciardiello, M.A., 2019. ENEA, a peach and apricot IgE-binding protein cross-reacting with the latex major allergen hev b 5. *Mol. Immunol.* 112, 347–357.
- Hedden, P., Sponsel, V., 2015. A century of gibberellin research. *J. Plant Growth Regul.* 34, 740–760.
- Inomata, N., Miyakawa, M., Aihara, M., 2017. Gibberellin-regulated protein in Japanese apricot is an allergen cross-reactive to Pru p 7. *Immun Inflamm Dis* 5, 469–479.
- Inomata, N., Miyakawa, M., Ikeda, N., Oda, K., Aihara, M., 2018. Identification of gibberellin-regulated protein as a new allergen in orange allergy. *Clin. Exp. Allergy* 48, 1509–1520.
- Klingebiel, C., Chantran, Y., Arif-Lusson, R., Ehrenberg, A.E., Östling, J., Poisson, A., Liabeuf, V., Agabriel, C., Birnbaum, J., Porri, F., Sarrat, A., Apoll, P.A., Vivinus, M., Garnier, L., Chiriac, A.M., Caimmi, D.P., Bourrain, J.L., Demoly, P., Guez, S., Boralevi, F., Lovato, B., Palussière, C., Leroy, S., Bourrier, T., Giovannini-Chami, L., Gouitaa, M., Aferiat-Derome, A., Charpin, D., Sofalvi, T., Cabon-Boudard, L., Massabie-Bouchat, Y.P., Hofmann, B., Bonardel, N., Dron-Gonzalez, M., Sterling, B., Carsin, A., Vivinus, S., Poitevin, B., Nicolau, L., Liautard, G., Soler, C., Mezouar, S., Annesi-Maesano, I., Mège, J.L., Lidholm, J., Vitte, J., Networks, a.t.A.a.A., 2019. Pru p 7 sensitization is a predominant cause of severe, cypress pollen-associated peach allergy. *Clin. Exp. Allergy* 49, 526–536.
- Li-jun, A.N., Liang, J.L.N., Chun-qin, Y., Tian-hong, L.L., 2008. Effect and functional mechanism of the action of exogenous gibberellin on flowering of peach. *Agri. Sci. China* 7, 1324–1332.
- Muhammad, I., Li, W.-Q., Jing, X.-Q., Zhou, M.-R., Shalmani, A., Ali, M., Wei, X.-Y., Sharif, R., Liu, W.-T., Chen, K.-M., 2019. a systematic in silico prediction of gibberellin acid stimulated GASA family members: a novel small peptide contributes to floral architecture and transcriptomic changes induced by external stimuli in rice. *J. Plant Physiol.* 234–235, 117–132.
- Nahirniak, V., Rivarola, M., Almasia, N.I., Barrios Barón, M.P., Hopp, H.E., Vile, D., Paniego, N., Vazquez Rovere, C., 2019. Snakin-1 affects reactive oxygen species and ascorbic acid levels and hormone balance in potato. *PLoS One* 14, e0214165.
- Palazzo, P., Tuppo, L., Giangrieco, I., Bernardi, M.L., Rafaiani, C., Crescenzo, R., Tamburrini, M., Zuzzi, S., Alessandri, C., Mari, A., Ciardiello, M.A., 2013. Prevalence and peculiarities of IgE reactivity to kiwifruit pectin methyltransferase and its inhibitor, act d 7 and act d 6, in subjects allergic to kiwifruit. *Food Res. Int.* 53, 24–30.
- Pasquariello, M.S., Palazzo, P., Tuppo, L., Liso, M., Petriccione, M., Rega, P., Tartaglia, A., Tamburrini, M., Alessandri, C., Ciardiello, M.A., Mari, A., 2012. Analysis of the potential allergenicity of traditional apple cultivars by multiplex biochip-based immunoassay. *Food Chem.* 135, 219–227.
- Sénéchal, H., Šantrůček, J., Melčová, M., Svoboda, P., Zidková, J., Charpin, D., Guilloux, L., Shahali, Y., Selva, M.A., Couderc, R., Aizawa, T., Poncet, P., 2018. A new allergen family involved in pollen food-associated syndrome: Snakin/gibberellin-regulated proteins. *J. Allergy Clin. Immunol.* 141, 411–414 e414.
- Shahali, Y., Sutra, J.-P., Charpin, D., Mari, A., Guilloux, L., Sénéchal, H., Poncet, P., 2012. Differential IgE sensitization to cypress pollen associated to a basic allergen of 14 kDa. *FEBS J.* 279, 1445–1455.
- Tamburrini, M., Cerasuolo, I., Carratore, V., Stanziola, A.A., Zofra, S., Romano, L., Camardella, L., Ciardiello, M.A., 2005. Kiwellin, a novel protein from kiwi fruit. Purification, biochemical characterization and identification as an allergen*. *Protein J.* 24, 423–429.
- Tuppo, L., Alessandri, C., Pomponi, D., Picone, D., Tamburrini, M., Ferrara, R., Petriccione, M., Mangone, I., Palazzo, P., Liso, M., Giangrieco, I., Crescenzo, R., Bernardi, M.L., Zennaro, D., Helmer-Citterich, M., Mari, A., Ciardiello, M.A., 2013. Peamaclein—a new peach allergenic protein: similarities, differences and misleading features compared to Pru p 3. *Clin. Exp. Allergy* 43, 128–140.
- Tuppo, L., Spadaccini, R., Alessandri, C., Wienk, H., Boelens, R., Giangrieco, I., Tamburrini, M., Mari, A., Picone, D., Ciardiello, M.A., 2014. Structure, stability, and IgE binding of the peach allergen peamaclein (Pru p 7). *Biopolymers* 102, 416–425.
- Tuppo, L., Alessandri, C., Pasquariello, M.S., Petriccione, M., Giangrieco, I., Tamburrini, M., Mari, A., Ciardiello, M.A., 2017. Pomegranate cultivars: identification of the New IgE-binding protein pommaclein and analysis of antioxidant variability. *J. Agric. Food. Chem.* 65, 2702–2710.
- Tuppo, L., Giangrieco, I., Alessandri, C., Ricciardi, T., Rafaiani, C., Ciancamerla, M., Ferrara, R., Zennaro, D., Bernardi, M.L., Tamburrini, M., Mari, A., Ciardiello, M.A., 2018. Pomegranate chitinase III: identification of a new allergen and analysis of sensitization patterns to chitinases. *Mol. Immunol.* 103, 89–95.