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Gibberellin-Regulated Protein sensitization in Japanese cedar (*Cryptomeria japonica*) pollen allergic Japanese cohorts

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Key words: Cry j 7; Japanese cedar pollen; pollen-food allergy syndrome; gibberellin-regulated protein; fruits

Abbreviation:

1-DE: One dimensional gel Electrophoresis; 2-DE: Two Dimensional gel Electrophoresis; AA: Amino-Acid; CD: Circular Dichroism; CPAP: Cupressaceae Pollen Allergic Patient; Cryj: *Cryptomeria japonica* (Japanese cedar); Cups *Cupressus sempervirens* (Mediterranean cypress); GASA: Gibberellic Acid Stimulated Arabidopsis; GRP: Gibberellin-regulated protein; IEF: Iso Electric Focusing; Juna: *Juniperus ashei* (Mountain cedar); LMW: Low Molecular weight; LTP: Lipid Transfer Protein; MS: Mass Spectrometry; NCa: activated NitroCellulose; NMR: Nuclear Magnetic Resonance; PBS: Phosphate Buffer saline; PFAS: Pollen Food Allergy Syndrome; PR: Pathogenesis Related; SPT: skin prick test, SDS-PAGE: Sodium Dodecyl Sulfate Poly Acrylamide Gel Electrophoresis.

Author contributions:

TM, YS, FR, JZ, XL performed the production of the recombinant allergens and physico-chemically characterized them. MT and ME recruited Japanese allergic patients and collected clinical and biological data. LGA and DC recruited European allergic patients and collected clinical and biological data. PC and CB performed bio- and physico-chemical analysis included mass spectrometry. HS, TA and PP conceived the study, participated in experimentation and wrote the manuscript. All authors exploited and discussed the results, read and corrected the various drafts of the manuscript before the submission of the final version.

To the editor,

Pollen-food allergy syndromes (PFAS) have been clinically reported and are based on structurally similar sensitizing allergens in pollen and plant food. Besides PR-10 or LTP allergens, the recently described Gibberellin-regulated proteins (GRP) are also allergens found in pollen and plant derived food, therefore are allergens involved in PFAS (1-2). They are found in Cupressaceae pollen and some fruits such as peach/apricot, citrus, pomegranate and cherry (3). The aim of the study was to compare GRP sensitization in two cohorts of Japanese children allergic to Japanese cedar (*Cryptomeria japonica*, Cryj) pollen: group 1 allergic to pollen only (n=21), group 2 allergic to pollen and fruit (n=22) suffering from systemic reaction (Table 1). Clinical data of recruited allergic patients were recorded at the department of pediatrics, Sagamihara National Hospital. Allergomics analysis was performed using direct or competitive IgE immunoblots after separation of the Japanese cedar pollen extracts in electrophoresis in 1 dimension (1-DE) or 2-DE followed by identification of allergens by mass spectrometry. Recombinant pollen and fruit GRPs were produced.

Associated fruit IgE-dependent allergy in group 2 mainly involved peach, citrus and apple but also cherry, Japanese apricot, strawberry and grape. In a cohort of fruit-only allergic children, the fruits were different, kiwi and banana (data not shown). IgE immunoblots showed a high heterogeneity from 95 to 10 kDa with couples of close bands in low molecular weight allergens (LMW, <30kDa) for group 2 (Figure1A). Dotline experiments for group 2 revealed that 10 patients out of 22 (46%) had IgE against rCry j 7 produced on the basis of the already reported sequence CJC5531 (<https://www.ffpri.affrc.go.jp/labs/cjgenome/members.html>), a GRP from *Cryptomeria japonica* male strobili (FigureS1, S2, S3). IgE reactivities against Pru p 7, Cit s 7, Pun g 7 or snakin-1, the GRPs from peach, orange, pomegranate or potato, respectively, were evidenced only in Cry j 7 positive patients who were all peach allergic (Figure1B). No GRP IgE binding was observed in dotlines of group 1 patients. Competitive inhibition of the IgE binding to Cryj pollen extract (Figure1C) with CJC5531/Cry j 7 abolished the binding to 2 LMW allergenic bands for 3 patients of group 2. A third allergenic LMW band for 2J17 patient and one for 1J11 patient as well as the high MW ones were not inhibited assessing the specificity of the inhibitions. The two isoforms of the native GRP detected were shown to be cationic when analysed in 2-DE immunoblots using 2J17+2J20 patients' IgE (Figure1D, spots 2 and 3), and to contain a peptide sequence of Cryj GRP using mass spectrometry (TableS2). A third LMW allergen recognized by the IgE from pollen-only allergic 1J19 patient, is acidic and corresponds to a 4-EF hand Ca-

binding protein, (Figure1D, spot 1 and TableS2) described allergenic in *Juniperus oxycedrus* pollen (4). The restriction of allergenic GRPs to Cupressaceae was already reported in 2012 (5) and documented herein. Cry j 7, Cup s 7 and Jun a 7, the GRPs from Cryj, *Cupressus sempervirens* (Cups) and *Juniperus ashei* pollen, respectively, cross-react (FigureS4, S5, S6) (6). However, in contrast to Cryj where several allergenic bands are present in LMW area, Cups shows only one, Cup s 7, either with cypress pollen allergic European patient (TableS1) or with cedar pollen allergic Japanese patient (FigureS7). Although all plants produce GRPs, allergenic GRPs are reported in few fruits and only in *Cupressaceae* pollen, therefore they cannot be considered as panallergens for the moment. Accessibility of the protein and interactions with insoluble components might vary between species. For instance 3D modelling of GRP revealed a cleft that should accommodate ligand(s) (FigureS8). In conclusion we showed that 46% of Cryj pollen Japanese allergic patients also allergic to fruits are sensitized to Cry j 7. This is the first study on the association of Cryj pollen and fruit GRPs sensitization in Japanese patient cohorts living in an area where Japanese cedars are growing.

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Table 1 : Clinical and biological available data of Japanese allergic patients used in the study. Group 1 CPAP pollen-only (n=21). Group 2 CPAP pollen-fruit (n=22). Allergy symptoms are noted as A: Asthma; AD: Atopic Dermatitis; AP: Abdominal pain; CAL: Change in activity level; C: Conjunctivitis; Co: Cough; D: Dyspnea; E: Emesis; F: Flushing; FEIA: Food-dependent exercise-induced anaphylaxis; GU: Generalized urticaria; I: Itching; LT: Laryngeal tightness; LU: Localized urticaria; N: Nausea; N/A: not available; POC: Prurit of oral cavity; R: Rhinitis; S: Sneezing; SE: Swollen Eyelids; W: Wheezing.

PATIENT			POLLEN sIgE (kUA/L)								FOOD							
Nr	Gender	Age year	Total IgE (kIU/L)	Japanese cedar	Japanese cypress	Birch	Ragweed	Orchard grass	Mugwort	Allergy symptoms	Fruits sIgE (kUA/L)	Type of FA symptom	SYMPTOMS					
													skin	eyes	oral cavity	Gastr o intesti nal	Respir atory	Ana phyla xis
Group 1																		
1J1	M	10	425	322.0	19.3	15.7	4.11	0.78	0.24	R	None	None						
1J2	M	14	373	99.9	11.8	1.33	0.16	0.19	0.10	R	None	None						
1J3	M	9	25200	822.0	19.8	19.1	5.45	38.5	3.86	AD, R	None	None						
1J4	F	8	5770	761.0	34.3	23.6	10.1	76.9	8.28	AD, R	None	None						
1J5	M	6	504	76.7	4.05	16.2	2.84	19.1	N/A	AD, R	None	None						
1J6	F	8	923	528.0	16.00	16.1	3.54	6.18	2.47	A, R	None	None						
1J7	M	5	245	66.1	8.16	14.7	0.43	0.12	0.12	R, C	None	None						

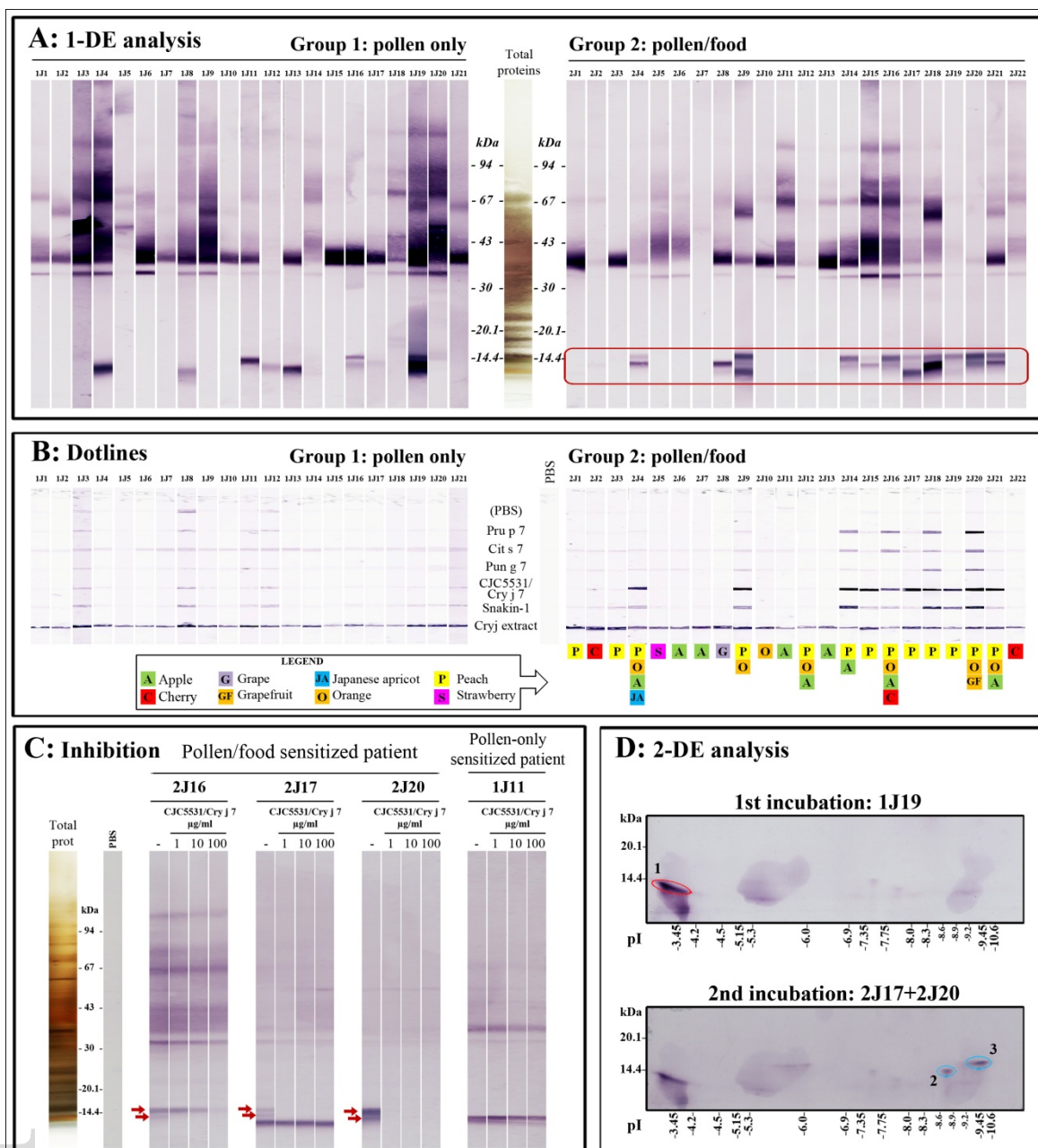
1J8	M	11	2870	77.2	15.2	43,00	4.45	5.33	3.82	AD, R, C	None	None					
1J9	M	14	2950	349.0	32.5	57.2	18	15.7	15.8	R	None	None					
1J10	M	9	801	96.4	15.3	1.89	9.34	34.8	0.33	A, R	None	None					
1J11	M	8	996	185.0	5.68	14.8	0.83	37.2	0.66	A, R, C	None	None					
1J12	F	6	1480	63.8	2;97	2.34	0.91	4.06	1.83	AD, R	None	None					
1J13	M	11	620	139.0	10.9	10.8	0.55	0.42	0.63	R, C	None	None					
1J14	M	12	1480	176.0	17.8	3.48	1.54	3.99	1.61	AD, R, C	None	None					
1J15	M	7	572	85.0	7.19	0.44	0.13	0.23	0.17	R	None	None					
1J16	M	10	545	99.6	16.5	<0.10	<0.10	0.10	<0.10	R	None	None					
1J17	F	8	258	45.6	1.94	0.17	0.23	0.29	<0.10	R	None	None					
1J18	M	6	153	41.3	2.68	0.8	4.92	1.49	0.41	AD, R, C	None	None					
1J19	F	8	1540	536.0	11.3	4.4	1.45	2.74	1.72	AD, R, C	None	None					
1J20	M	8	853	291.0	10.3	17.2	15.1	7.47	3.73	R	None	None					
1J21	M	8	380	273.0	7.84	1.16	0.84	4.47	0.29	AD, R, C	None	None					
Group 2																	
2J1	F	16	4310	219.0	N/A	58	1.93	14.8	N/A	AD, R, C	Peach: 15.2	Immediate					
2J2	F	16	2630	79.1	N/A	0.19	0.32	0.35	0.42	A, AD, R, C	Cherry: 0.17	Immediate	D				
2J3	M	12	1880	477.00	N/A	N/A	N/A	N/A	N/A	A, AD, R	Peach: 7.17	Immediate					
2J4	M	9	54.4	40.3	N/A	0.37	<0.35	1.31	N/A	R, C	Japanese apricot: N/A Apple: <0.35 Peach: 0.58 Orange: 0.49	Immediate	SE, I	C	POC	S, Co, D, W	1

2J5	M	9	985	76.3	N/A	N/A	N/A	N/A	N/A	A, AD, R	Strawberry: 0.98	Immediate	F			W, D	1
2J6	M	4	170	44.9	N/A	24.1	N/A	N/A	N/A	R	Apple: 2.53	Immediate	F, I	POC	AP		1
2J7	M	16	1230	35.1	5.76	N/A	3.4	0.33	N/A	A, R	Apple: 2.94	Immediate	LU				
2J8	M	9	870	>100	N/A	2.4	N/A	N/A	N/A	R	Grape: N/A	Immediate		POC		D	
2J9	F	17	220	146.0	N/A	0.11	N/A	N/A	N/A	R	Peach: 0.25	Immediate	GU, SE	POC		Co,	1
											Orange: 0.41					CAL	
2J10	M	17	1210	577.0	37.80	200	N/A	N/A	N/A	AD, R, C	Orange: 0.22	FEIAn	LU	C	LT	E, AP	1
2J11	M	11	1120	186.0	N/A	54.3	2.52	40.5	N/A	A, R, C	Apple: 6.44	Immediate	I	POC	E	Co, W	1
2J12	M	12	78	27.8	N/A	<0.35	<0.35	N/A	N/A	A, R, C	Orange: N/A	Immediate	GU	C			1
											Peach: N/A	/FEIAn	I				
											Apple: <0.35						
2J13	M	16	2270	88.9	N/A	10.6	9.51	175	6.42	AD, R, C	Apple: 0.46	Immediate				AP	
2J14	M	13	415	231.0	N/A	0.34	0.24	4.2	N/A	R	Peach: 3.44	Immediate	GU		E	D	1
											Apple: 0.29						
2J15	M	15	197	62.6	13.1	1.89	0.28	3.55	1.08	R	Peach: 1.53	Immediate	GU				
2J16	M	15	378	21.9	3.62	1.96	0.89	3.58	1.47	A, R, C	Cherry: N/A	Immediate	GU	LT		W, Co,	1
											Peach: 2.59	/FEIAn				S	
											Apple: 1.3						
											Orange: 2.35						
2J17	F	6	1580	223.0	N/A	0.51	N/A	N/A	N/A	R	Peach: 1.05	Immediate	GU				
2J18	M	15	1160	344,0	N/A	0.74	N/A	N/A	N/A	A, R, C	Peach: 3.74	FEIAn	GU		N	D	1
2J19	M	18	193	24.0	N/A	<0.10	N/A	N/A	N/A	AD, R, C	Peach:<0.10	Immediate		C	AP	S	

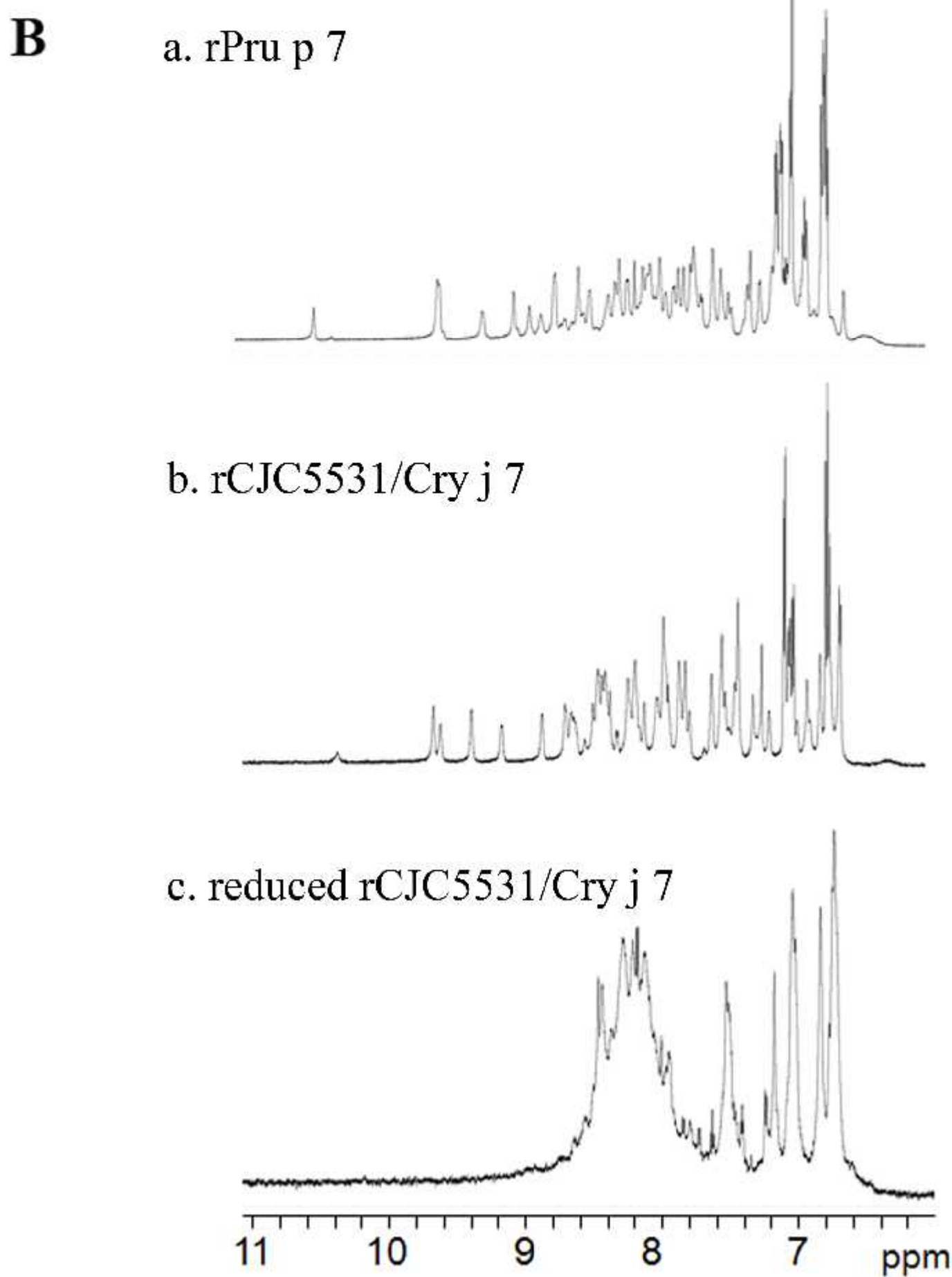
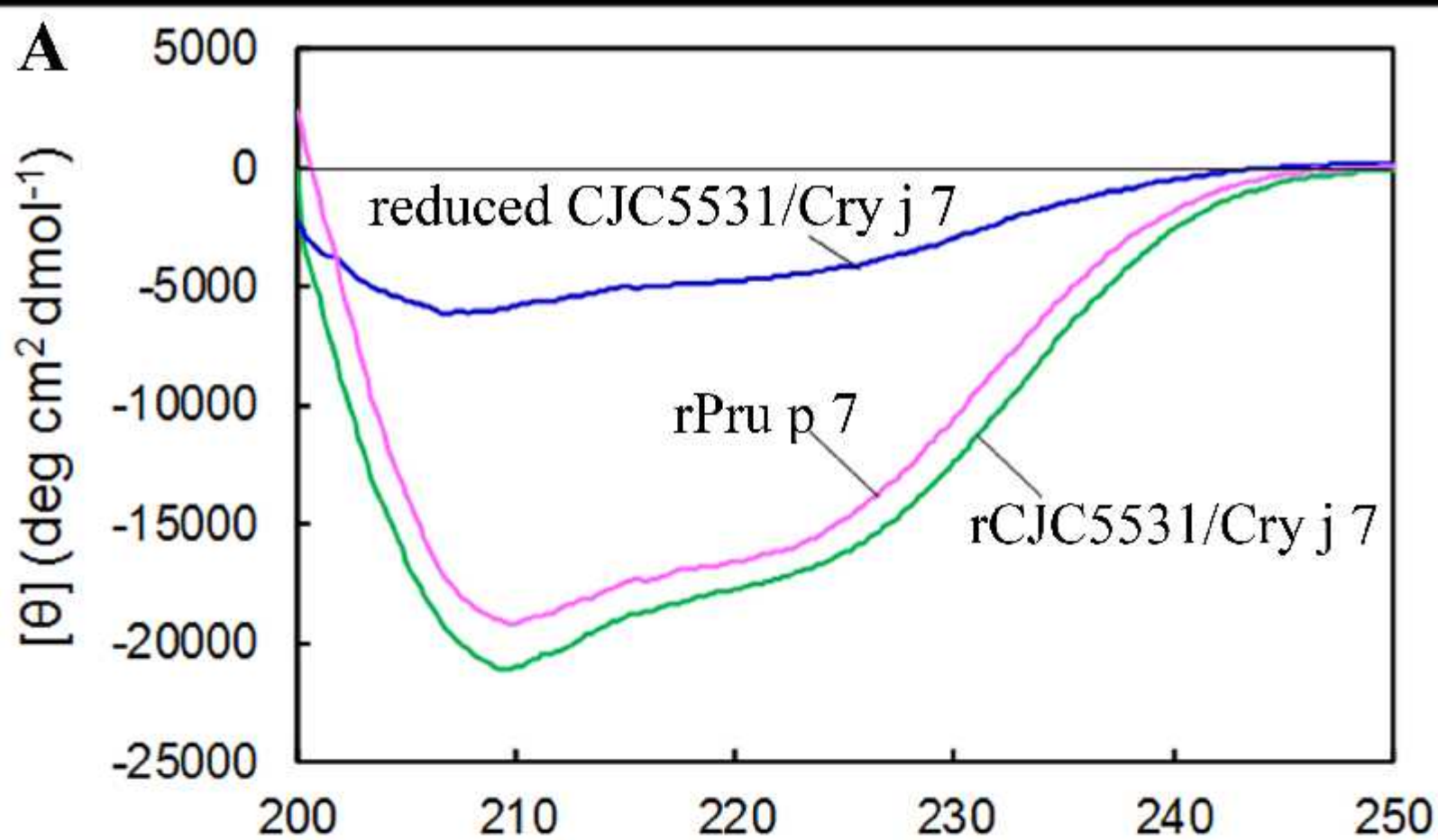
2J20	M	9	598	222.0	19.6	N/A	0.76	N/A	0.45	Unknown	Grapefruit: 2.29 Peach: 6.45 Orange: 1.51	Immediate /FEIAn	I, SE	LT	AP	S, CAL	1
2J21	F	17	646	129.0	N/A	2.35	2.26	1.25	0.97	R, C	Peach: 3.96 Apple: 2.07 Orange: 4.99	Immediate	F, GU	LT, POC	Cramps, AP	Co	1
2J22	F	6	1770	173.0	N/A	1.44	1.13	1.18	N/A	AD, R	Cherry: N/A	Immediate	C	POC	E	Co	1

Legend to Figure 1:

IgE immunoreactivity of Japanese patients against Cryj pollen. Total proteins are silver stained. PBS: no serum. A: 1-DE IgE immunoreactivity. Red frame highlights couples of close IgE reactive bands in LMW area B: Dotlines immunoassay with allergenic sources. Fruit allergies are indicated with a letter and a color code. C: Competitive inhibition of the IgE binding against LMW allergens by rCJC5531/Cry j 7. Group 2: 3 patients, group 1: 1 patient. Arrows indicate the inhibited IgE reactive bands. D: 2-DE IgE immunoreactivity to LMW Cryj proteins. First incubation: 1J19 patient's serum. Second incubation: 2J17+2J20 patient's sera. Red ellipse (spot 1): allergen recognized by 1J19 patients's IgE. Blue ellipses: allergens recognized by 2J17+2J20 patients' IgE (spots 2 and 3).



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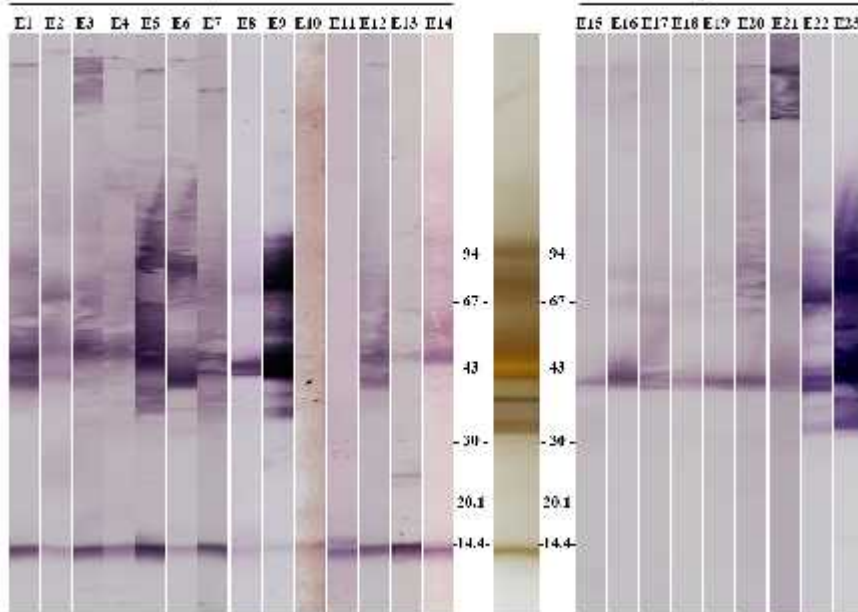


A

Cupressus sempervirens

Cup s 7 +

Cup s 7 -



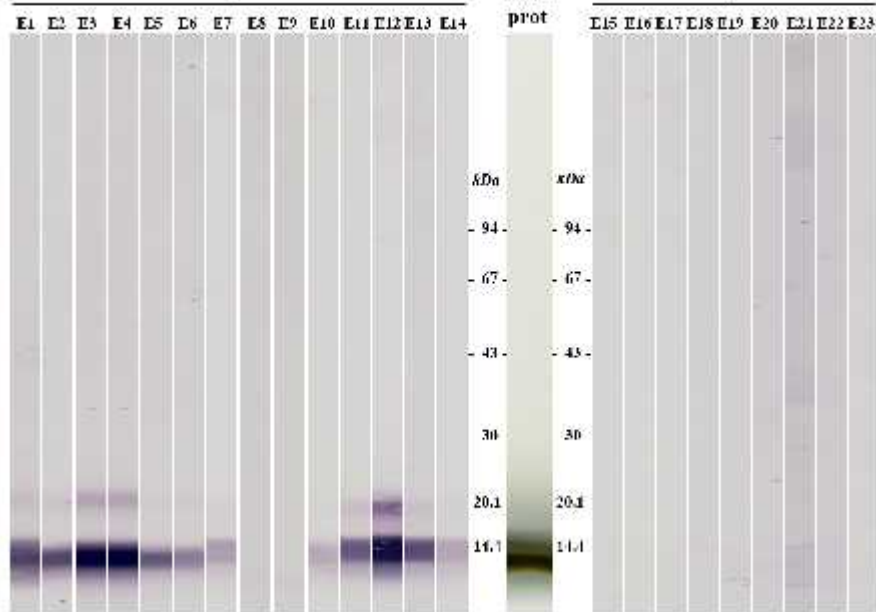
B

rCJC5531/Cry j 7

Cup s 7 +

Total
prot

Cup s 7 -



E14**E1****E12****E5****E16**Label
Protein

PDS

CJC5531-Cy7
($\mu\text{g/ml}$)CJC5531-Cy7
($\mu\text{g/ml}$)CJC5531-Cy7
($\mu\text{g/ml}$)CJC5531-Cy7
($\mu\text{g/ml}$)CJC5531-Cy7
($\mu\text{g/ml}$)

Caps 1/10

Caps 1/10

Caps 1/10

Caps 1/10

Caps 1/10

kDa

94

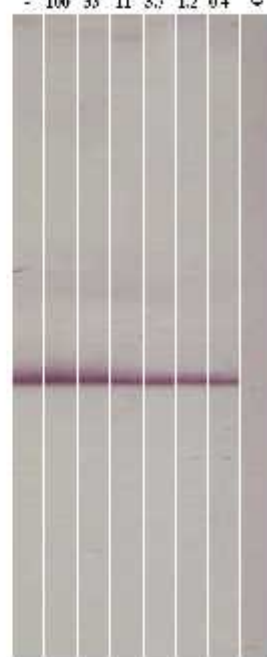
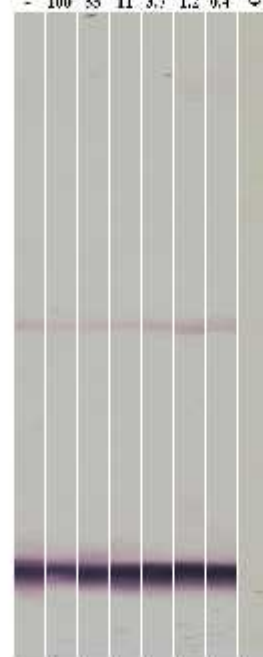
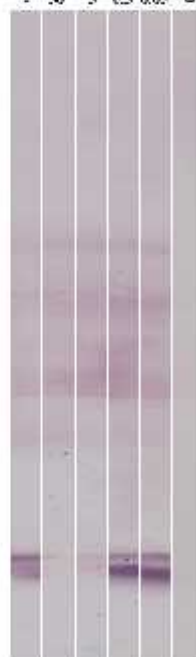
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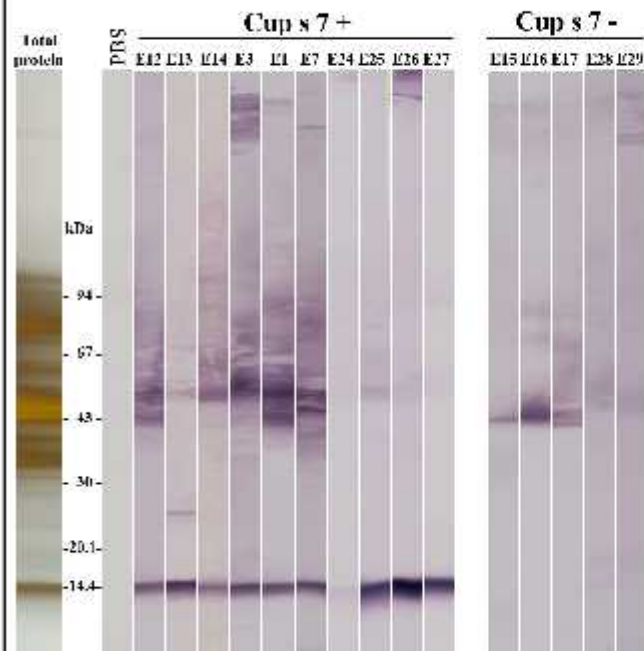
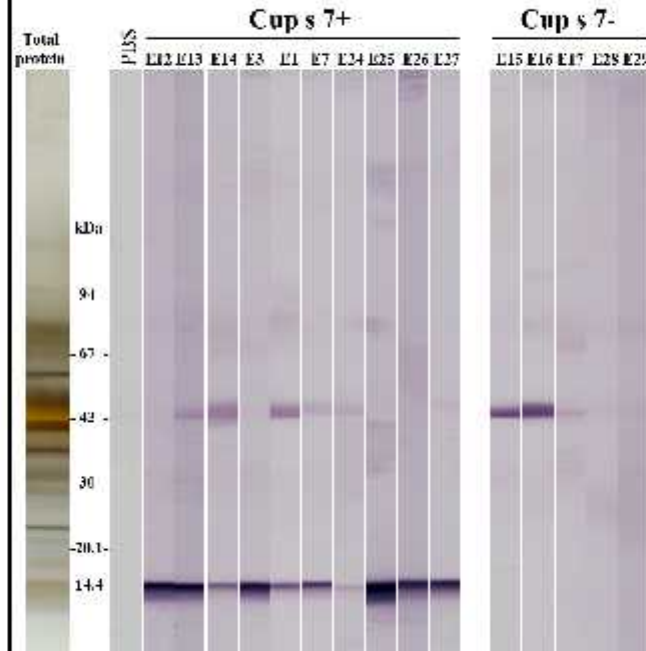
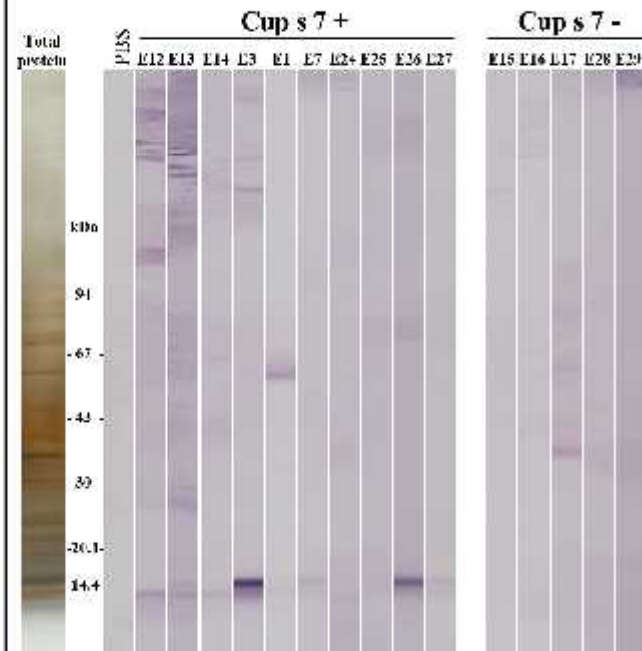
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30

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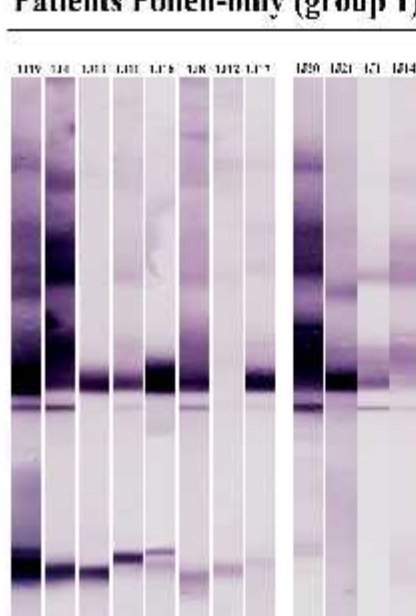
14.4



A*Cupressus sempervirens***B***Juniperus ashei***C***Cryptomeria japonica*

A Patients Pollen-only (group 1)

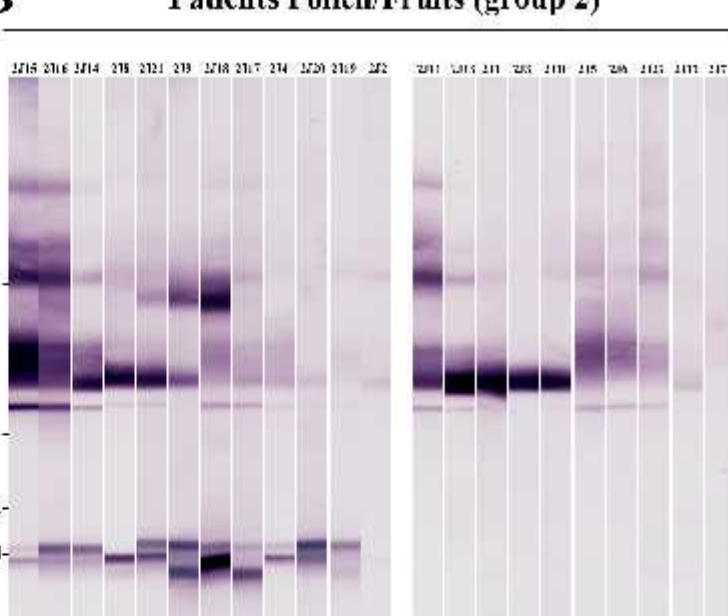
Cryj extract



B Patients Pollen/Fruits (group 2)

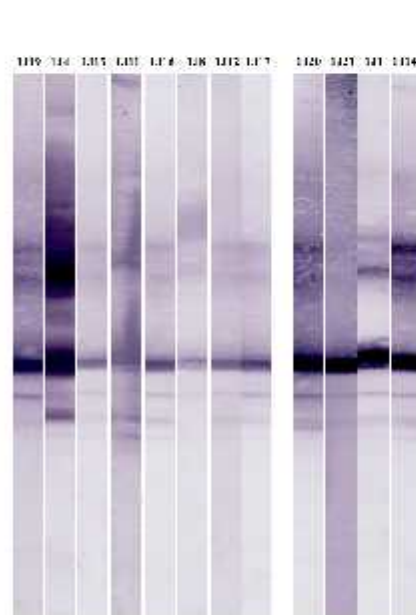
Cryj
total
proteins

94
-67-
43
-30-
-20.1-
-14.4-



C

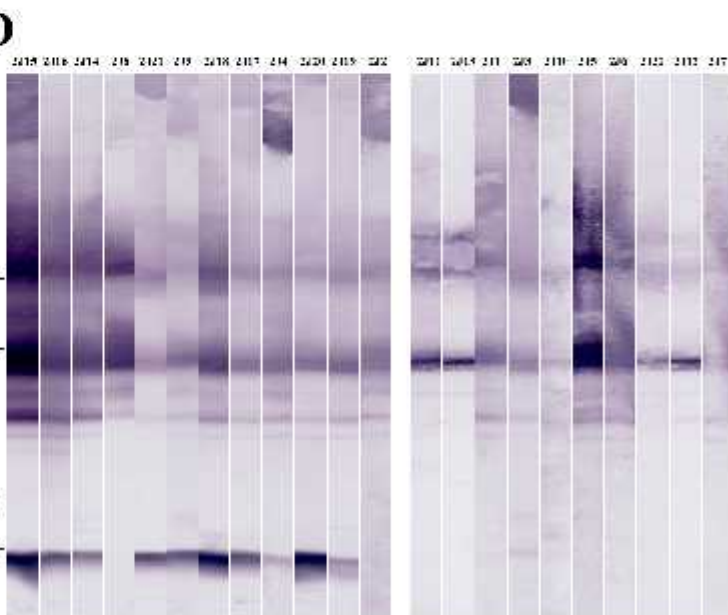
Cups extract



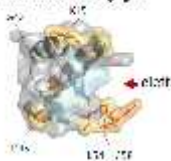
Cups
total
proteins

94
-67-
43
-30-
-20.1-
-14.4-

D



CJC'5531/Cry j 7



Prup p 7



Cit s 7



Pun g 7

