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Review

Central and Eastern European Spring Pollen Allergens and Their Expression Analysis—State of the Art

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Abstract: Spring pollinosis has become a part of life for many people throughout the world. A wide range of knowledge about the allergenic potential of individual pollen allergen types is documented well, but the starting point of the pollen allergens expression regulation in plants itself is still not fully answered. Expression analysis of pollen allergens does not yet have any specific protocols or methods developed, despite a very good sequence background available in public bioinformatics databases. However, research in this area of interest has a great application potential for breeding and biotechnology of allergenic plants that may benefit from the knowledge of the expression of allergen coding genes in individual varieties or genotypes. Here, a brief review of up-to-date knowledge about the coding sequences of central and eastern European spring pollen allergens is introduced together with real-time based analysis of the expression of two of the main pollen allergens–PR protein type and profilin type of birch and hazelnut.

Keywords: spring pollinosis; allergen expression; Bet v1; profilin; real-time PCR

1. North, Central and Eastern Europe Spring Pollinosis Sources

Bronchial asthma, allergic rhinitis and allergic conjunctivitis—these are some of symptoms of pollinosis. Seasonal pollen allergy is the most common allergic disease with the significantly increasing tendency. Nowadays, it affects up to one fifth of the population and it can occur at any time during life, but most often between the 15th and 25th year. Whether the allergy appears during life, is an interaction among heredity factors, intestinal microflora, nutrition and the length of time breast-fed [1].

The most important cause of the IgE-mediated allergy is pollen and its proteins from trees and grasses [2]. When thinking of trees, not only is pollen the source of allergens, but also fruits and seeds. In this review, the main objective is a summarization of the current knowledge and already developed methods for the analysis of the expression of pollen allergens per se, directly in pollen. Analyses such as these are important, especially when considering that every pollinosis season differs when compared to previous ones. As will be seen further in the text, not only the amount of pollen in the atmosphere, which is strongly dependent on the weather conditions, but also the place of the growth decides the level of allergen expression, i.e., releasing of allergenic molecules. Variations exist in allergen content in pollen grains and, moreover, allergens are also released via submicroscopic particles [3,4]. This is why quantifying different aspects of allergen characteristics has become realized [5].

The most allergenic tree pollen is produced in spring in Europe by alder, hazel, yew, elm, willow, ash, birch, plane and poplar. The main period of pollen release with peak periods depends on the region.

Alder and hazel are the first to flower (December–April) followed by willow and elm (February–April), then ash and birch (March–May) and finally poplar (March–April). All of these trees collectively are the most important source of European spring pollinosis (Table 1) and the main allergens of the highly allergenic trees are well defined at the protein level and accessible in different allergen databases, such as WHO/IUIS (World Health Organization/International Union of Immunological Societes) Allergen Nomenclature (www.allergen.org); Allergome (www.allergome.org); SDAP (Structural Database of Allergenic Proteins) (www.fermi.utmb.edu); AllFam (www.meduniwien.ac.at) or Allergen Online (www.allergenonline.org). Almost all known tree pollen allergens are low-molecular weight intracellular proteins or glycoproteins and are released after touching an aquatic surface such as human mucous membranes. Many comprehensive descriptions of the structural characteristics of individual functions of pollen allergenic proteins are given in the literature [6,7], which is why only a very brief description of the main allergenic proteins is given here in Table 1. They exist in different trees as proteins with significant sequence homology and cross-reactive epitopes [8].

Table 1. Allergenic proteins characteristics of main European spring pollinosis.

Allergen Type/Number	Occurrence of Homologs	Biological Function
Bet v1 type	alder, hazel [1], birch	pathogenesis related protein [8]
Profilin (Bet v2 type)	hazel, birch	actin-binding protein [8]
EF hands type	alder, birch	Ca^{2+}-binding protein [8]
olive pollen allergen	ash, London plane tree	unknown [9]
cyclophilin	birch, oriental plane	immunosuppressant receptors–immunophilins [9–11]
lipid transfer protein	Hazel [2], London plane tree, oriental plane	lipid transport [8]

[1] Cor a 1 of hazel is refereed to and belongs to the thaumatin-like protein by [12] and AllFam database; [2] Cor a 8 of hazel is refereed to and belongs to the prolamin protein family by [13] and AllFam database.

2. Spring Tree Pollinosis—Actual Allergen Nucleic Acids Sequences and Function Knowledge

Pollen from different species of monocotyledonic or and dicotyledonic plants is regarded to be the most frequent and active allergen source. Pollen allergens are low molecular weight proteins or glycoproteins and are located intracellular in the pollen grains. Their physiological activation starts after pollen hydration on mucosal surfaces or by liberation from small allergen containing respirable particles [14].

Currently, it is still in most cases impossible to purify and describe all of the major and minor allergens of natural allergen sources that are active in aqueous buffers. Thanks to the advance in the field of molecular genetics, this problem can be overcome by using cDNAs for recombinant production of allergens from their sources [8].

Obtaining the allergen-encoded cDNAs, three different methods are applied. The first is based on immunoscreening, the second is based on amino acids determination, and the third usesthe sequence similarity at the protein and nucleic acid level. The workflow for the first one is: mRNA isolation from allergenic pollen → cDNA synthesis → construction of expressed cDNA library → IgE immunoscreening → sequencing. Today, using this method together with robotic-based screening provides cost effective and rapid identification of allergens, even from complex allergenic sources [15]. The workflow for the second is: Allergen protein purification → determination of amino acid composition and N-terminal amino acid sequence → oligonucleotides design → PCR cloning or screening of cDNA libraries → sequencing. The workflow for the third is: Known cDNA sequences and amino acids sequences comparison → degenerate primers design → the first-strand cDNA amplification and amplification of 5′ cDNA ends → cloning and sequencing of cDNA. Comparing them to the immunoscreening method, the PCR based technology is much more effective and the growing amount of data stored in public databases of allergens facilitate allergen cloning using this approach [16]. Described methods was used for the isolation of cDNA of following spring pollen allergens: Bet v1, Bet v2, Bet v3, Bet v4, Aln g 4, Cor a 1 and Aln g 1 [16–20]. When considering

north, central and eastern Europe, the most allergenic tree pollen is produced by the Order of Fagales. It comprises the three main allergenic families—Betulaceae, Corylaceae and Fagaceae. Pollen allergens that are known for their genera are listed in Table 2.

Table 2. Accession codes of nucleotide sequences for spring pollinosis allergens.

Species	Pollen Allergen *	Nucleotide Accession Code	Type of Nucleic Acid	Function or Similarity *
Alnus glutinosa	Aln g 1	S50892.1	mRNA	PR10; Bet v1 type
	Aln g 4	Y17713.1	mRNA	Ca^{2+}-binding protein
Corylus avellana	Cor a 1 [a]	Z72440.1 Z72439.1	DNA	PR 10; Bet v1 type
	Cor a 2	AF327623.1 AF327622.1	mRNA	profilin; Bet v2 type
	Cor a 8 [b]	AF329829.1	mRNA	lipid transfer protein precursor
	Cor a 9 [b,c]	KF494372	mRNA	11S globulin (legumin-like)
	Cor a 10	AJ295617.1	mRNA	luminal binding protein
	Cor a 11 [b]	AF441864.1	mRNA	11S globulin (vicilin-like)
	Cor a 12 Cor a 13	AY224599	mRNA	oleosin
	Cor a 14 [b]	FJ358504	mRNA	2S albumin
Fraxinus excelsior	Fra e 1 [d]	AY377127.1	mRNA	Ole e 1 related (glycosylated protein)
	Fra e 2	KC920922.1	mRNA	profilin
	Fra e 3	KC920923.1	mRNA	Ca binding protein
	Fra e 6	KC920921.1	mRNA	not identified
	Fra e 9	KC920916.1	mRNA	not identified
	Fra e 10	KC920924.1	mRNA	not identified
	Fra e 11	KC920915.1	mRNA	not identified
	Fra e 12	EF626802.1	mRNA	not identified
Betula verrucosa	Bet v1 [e]	X15877.1	mRNA	PR10
	Bet v2	M65179	mRNA	profilin
	Bet v3	X79267	mRNA	Ca^{2+}-binding protein
	Bet v4	X87153	mRNA	Ca^{2+}-binding protein
	Bet v5	-	-	isoflavonereductase
	Bet v6	AF135127 AF282850	mRNA	isoflavonereductase
	Bet v7	AJ311666.1	mRNA	cyclophilin
	Bet v8	-	-	pectin esterase
Platanus acerifolia	Pla a 1	AJ427413.2	mRNA	invertase inhibitor
	Pla a 2	AJ586898.1	mRNA	polymethylgalacturonase
	Pla a 3	-	-	Lipid transfer protein
	Pla a 8 Ole e 1	KM397755.1	mRNA	pollen allergen
Platanus orientalis	Pla or 1	EU296476.1	mRNA	plant invertase/pectin methylesterase inhibitor (cyclophilin)
	Pla or 2	EU296477	mRNA	polygalacturonase
	Pla or 3	EU296478	mRNA	lipid transfer protein precursor

* As summarized in [8]; [a] isoallergens mRNA sequences of Cor a 1 stored in NCBI under the accessions: AF323973-5, AF136945, X70997-9, and X71000; [b] referred to as non-pollen related allergens in [21]; [c] another 11S globulin-like protein mRNAs are stored in NCBI under the accessions: JN67437-9, and AF449424; [d] isoallergens mRNA sequences of Fra e1 stored in NCBI under the accessions: AY652744.1, and AF526295.1; [e] in total, 52 isoforms are stored in SDAP database.

3. Birch Pollen Allergens—Actual DNA/RNA Sequence Data

Bet v1: A major birch allergen originated in *Betulla verrucosa* is actually the most known and described as one with in total three subfamilies that were identified by their sequences similarity comparison [22]. All three subfamilies are abundant in vascular plants. The first, pathogenesis-related protein family PR-10, is expressed in plants following the signals as pathogen attack or abiotic stress. They are most concentrated in reproductive tissues—pollen, seeds and fruits [23]. This birch allergen protein was identified to posses a very similar structure with the human lipocalin 2 [24]. Both were invented to have the specific structures that allowed them to bind iron and only in the situation when no iron is binding on birch Bet v1 protein it becomes an allergen by the manner of affecting of Th2 cells of the human immune system [24]. Park et al. [25] reported the ribonuclease activity of pathogenesis-related proteins with the function in antiviral pathway. The other subfamily of Bet v1

allergens was described as a group of major latex proteins and ripening-related proteins in the latex of opium poppy [26,27]. The last of the Bet v1 subfamilies comprises proteins containing members with S-norcoclaurin synthase activity and are involved in alkaloid biosynthesis [28].

In total, nine Bet v1 isoforms are known as to be naturally occurring, a, b, c, d, e, f, g, j and i. When regarding the induction of type I allergic symptoms, they differ among themselves by the ability to react with the IgE from patients and it is reported [29] that the IgE binding activity, when compared in vitro and in vivo, is characterized directly by the six amino acid residues at different positions of the Bet v1 molecule. *Betula verrucosa/pendula* major pollen allergen is well defined on the nucleotide level and, in total, 47 isoallergens sequences are available in the NCBI database for mRNA with the sequence identities. Here, the conserved parts were obtained first by megablast algorithm and, subsequently, the nucleotide differences were found among them as summarized in Figure 1. The fylogenetic similarity of the sequences of Bet v1 isoallergens with the gene coding for the major birch pollen allergen Betv1 (X15877.1) is illustrated in the dendrogram constructed by NJ procedure (Figure 2).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	45	46	47	48
1	0																																														
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6	3	28	6	15	12	0																																									
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8	1	29	8	13	12	2	3	0																																							
9	15	17	20	9	2	14	13	14	0																																						
10	69	39	40	39	35	35	35	33	35	0																																					
11	51	29	43	42	38	38	38	36	38	10	0																																				
12	32	26	30	34	30	24	25	24	31	55	50	0																																			
13	27	29	24	20	14	18	17	19	12	52	55	36	0																																		
14	36	25	29	33	29	23	24	23	30	54	50	1	35	0																																	
15	33	30	23	11	5	17	16	17	3	66	52	41	17	42	0																																
16	38	26	30	34	30	24	25	24	31	56	51	1	37	2	42	0																															
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18	28	24	19	13	7	13	12	13	5	64	50	36	12	37	5	37	40	0																													
19	68	40	44	43	39	39	39	37	39	15	7	55	60	55	64	56	56	62	0																												
20	35	32	29	15	12	23	23	21	10	78	43	41	23	41	12	42	44	13	56	0																											
21	87	47	50	49	47	45	45	45	45	76	49	63	68	63	69	63	64	68	63	73	0																										
22	84	50	53	52	50	48	48	48	48	77	51	65	71	65	71	65	66	70	63	76	14	0																									
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24	21	21	28	15	12	22	22	20	10	29	28	35	22	34	13	35	35	15	29	8	43	46	*	0																							
25	15	37	21	26	27	18	16	16	27	27	26	33	31	32	30	33	35	26	27	27	43	46	*	26	0																						
26	9	35	14	21	18	8	9	8	20	41	44	29	24	28	23	29	31	19	45	29	51	52	*	28	24	0																					
27	19	31	36	29	26	30	30	28	24	16	14	40	31	39	27	40	40	26	15	22	39	42	*	14	24	36	0																				
28	6	31	9	18	15	3	6	5	17	38	41	27	21	26	20	27	29	16	42	26	48	51	*	25	21	11	33	0																			
29	7	27	12	15	10	6	7	6	8	32	35	25	12	24	11	25	27	7	36	17	42	45	*	17	20	12	25	9	0																		
30	16	32	22	15	17	17	18	15	17	28	31	34	29	33	20	34	36	22	32	24	46	49	*	23	13	23	37	20	19	0																	
31	8	28	15	8	11	11	10	9	9	37	40	32	19	31	12	32	34	14	41	16	48	51	*	15	21	17	26	14	11	16	0																
32	10	22	15	24	19	11	10	11	19	41	41	17	19	16	24	17	19	20	42	26	48	51	*	25	21	17	33	14	13	26	18	0															
33	8	36	13	22	19	9	8	9	21	42	45	30	25	29	24	30	32	20	46	30	52	55	*	29	23	1	37	12	13	24	16	16	0														
34	6	32	9	20	9	7	4	7	11	34	37	27	15	26	14	27	29	10	38	21	46	49	*	21	20	23	29	10	5	22	14	12	12	0													
35	11	27	18	5	8	12	13	10	6	37	40	31	18	30	9	31	33	11	41	13	46	49	*	12	24	18	26	15	12	13	5	21	19	17	0												
36	3	27	9	17	16	5	5	4	18	37	36	24	22	23	21	24	26	17	37	21	45	48	*	20	14	12	28	8	10	19	11	9	11	9	14	0											
37	5	35	12	19	18	8	7	6	20	39	40	28	24	27	23	28	30	19	41	25	48	47	*	26	20	14	34	11	12	21	13	15	13	11	16	8	0										
38	6	31	9	18	13	3	4	5	15	37	38	25	19	24	18	25	27	14	39	23	45	48	*	24	20	11	32	6	9	22	14	14	12	8	15	8	7	0									
39	11	36	18	17	20	14	13	12	15	42	45	33	22	32	21	33	34	17	46	25	44	46	*	23	24	20	21	17	14	25	11	21	19	17	14	14	12	17	0								
40	10	36	17	15	19	13	12	11	14	42	45	34	19	33	18	34	36	14	46	22	52	48	*	23	23	19	31	16	13	24	10	20	18	16	13	13	12	15	11	0							
41	5	27	10	13	12	4	5	4	12	34	37	23	16	22	15	23	25	11	38	21	43	46	*	20	18	10	29	7	6	15	11	13	11	9	10	8	10	7	14	13	0						
42	11	27	18	4	18	12	13	10	6	37	40	31	16	30	7	31	33	9	41	11	47	50	*	12	24	18	26	15	12	13	5	21	19	17	2	14	16	15	14	11	10	0					
43	2	30	7	16	13	3	2	3	15	36	39	25	19	24	18	25	27	14	40	24	46	49	*	23	17	9	31	6	7	18	10	10	8	6	13	5	7	6	11	12	5	13	0				
44	1	29	6	15	12	2	1	2	14	35	38	24	18	23	17	24	26	13	39	23	45	48	*	22	16	8	30	5	6	17	9	9	7	5	12	4	6	5	12	11	4	12	1	0			
45	6	35	13	20	19	9	8	7	21	39	40	29	24	28	24	29	30	20	41	26	49	48	*	26	21	15	34	12	13	22	14	16	14	12	17	9	4	9	11	12	11	17	8	7	0		
46	75	38	42	39	37	37	37	35	37	23	7	49	54	49	73	50	50	71	20	75	74	75	*	29	25	43	17	30	34	30	39	40	44	36	39	35	39	37	44	44	36	39	38	37	39	0	
47	77	38	42	39	37	37	37	35	37	17	7	49	54	49	71	50	50	69	18	65	69	68	*	29	25	43	17	40	34	30	39	40	44	36	39	35	39	37	44	44	36	39	38	37	39	2	0

Figure 1. Numbers of nucleotide differences among Bet v1 isoforms for the conserved part based on the NCBI data. * No sequence homology found; all the isosoforms are compared to: X15877.1 and the sequences are coded as follow: 1, AF124839.1; 2, AF124838.1; 3, AF124837.1; 4, AJ002110.1; 5, AJ002109.1; 6, AJ002108.1; 7, AJ002107.1; 8, AJ002106.1; 9, X77200.1; 10, X77272.1; 11, X77274.1; 12, X77273.1; 13, X77271.1; 14, X77270.1; 15, X77268.1; 16, X77267.1; 17, X77266.1; 18, X77265.1; 19, X77269.1; 20, X77599.1; 21, X77600.1; 22, X77601.1; 23, Y12560.1; 24, AJ006915.1; 25, AJ006914.1; 26, AJ006913.1; 27, AJ006912.1; 28, AJ006911.1; 29, AJ006910.1; 30, AJ006909.1; 31, AJ006908.1; 32, AJ006907.1; 33, AJ006905.1; 34, AJ006904.1; 35, AJ006903.1; 36, AJ006906.1; 37, Z80106.1; 38, Z80105.1; 39, Z80103.1; 40, Z80102.1; 41, Z80101.1; 42, Z80100.1; 43, Z80099.1; 44, Z80098.1; 45, Z80104.1; 46, X82028.1; and 47, X81972.1.

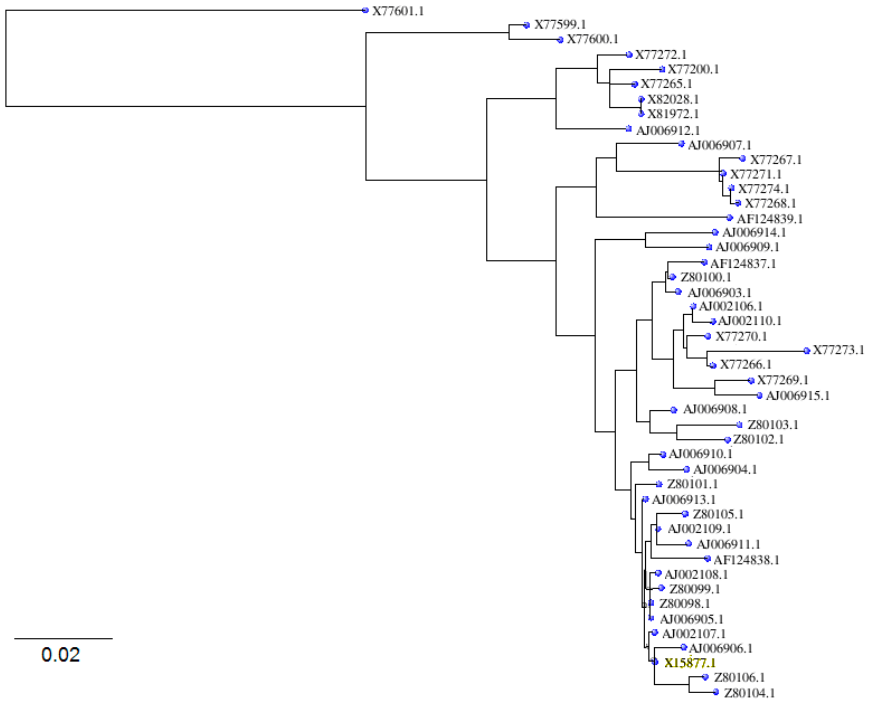

Figure 2. Sequences fylogenesis of Bet v1 isoallergens. The isoforms correspond with the number codes in Figure 1.

4. Minor Central and Eastern Pollen Allergens—Actual DNA/RNA Sequence Data

Closely-related and cross-reactive allergens with the Bet v1 were found in the pollen of some other trees from the order Fagales such as *Corylus avellana*, *Alnus glutinosa* or *Castanea sativa* (Table 2). For this allergen, the IgE cross-reactivity among Bet v1 and homologous allergens from fruits or vegetables that belong to the Rosaceae, Apiaceae or Fabaceae is often described [22].

Fra e 1 is amain pollen allergen of ash that is widely distributed in both central and northern Europe. The clinical relevance of the ash pollen allergen is quite difficult to value because its pollination overlaps with that of Betulaceae [30]. Currently, three main isoforms of Fra e 1 are stored in the sequence database with the following accession numbers: AY652744.1, AY377127.1 and AF526295.1. All of them are for mRNA. The sequence identity between AY652744.1 and AF526295.1 is 99%. In total, four differences exist in their sequences: in AF526295.1 G is replaced by C in the 135th position, C is replaced by G in the 271st position, G is replaced by A in the 300th position and C is replaced by T in the 380th position. The sequence identity between AY652744.1 and AY377127.1 is 89% with the differences listed in the Table 3.

Table 3. Nucleotide differences between ash Fra e 1 allergen mRNA isoforms.

Isoform/Nucleotide Position *	**1**	**2**	**3**	**27**	**27**	**33**	**34**	**36**	**37**	**55**	**73**	**85**	**96**
AY652744.1	G	A	G	T	T	C	T	C	G	T	G	G	A
AY377127.1	x	x	x	C	A	T	C	T	A	C	C	A	G
isoform/nucleotide position *	102	103	104	108	112	131	135	148	150	165	166	182	203
AY652744.1	T	C	A	C	G	A	G	A	A	A	G	C	G
AY377127.1	A	A	C	T	A	G	C	G	C	G	A	G	T
nucleotide position *	206	208	214	235	240	241	242	243	244	255	256	264	271
AY652744.1	A	T	C	C	T	G	T	A	C	C	A	A	C
AY377127.1	G	G	T	T	C	A	C	G	T	T	G	G	A
isoform/nucleotide position *	284	285	300	306	309	324	339	348	391	397	409	416	-
AY652744.1	C	C	G	G	G	C	C	G	A	C	T	T	-
AY377127.1	T	T	A	A	A	T	A	C	C	G	C	C	-

* As in the sequence of AY652744.1.

Aln g 1, the major allergen in pollen of alder, is also a member of the Bet v1 family. Only a sequence of mRNA actually exists in the public databases with the accession number S50892. Valenta et al. [31] has performed the alignments among the selected major allergens— Bet v1, Aln g 1 and Cor a 1. In hybridization, similar binding pattern of Bet v1 cDNA probe was detected to pollen RNAs from all of the analyzed species. This confirms the high homology of the mRNAs coding for the analyzed allergens. Based on these observations, an extract with only one major allergen from those analyzed should be sufficient for diagnostic and therapeutic purposes, as the authors have concluded [31].

5. Hazelnut Pollen Allergens—Actual DNA/RNA Sequence Data

Avery different bioinformatics situation exist for *Corylus avellana* major allergen Cor a 1. Currently, 1649 profilin sequences for different plant species are available at NCBI GenBank database and about half of them were isolated from pollen. Jimenez-Lopez et al. [7] reported in their study that no data regarding interspecific comparisons and cultivars sequence variability is available for pollen profilins in the literature and the presence of polymorphism is described in a low number of sequences in different plant species.

For hazel pollen profilin, in total eight isoallergens for mRNA and two sequences for DNA on the nucleotide level sequences are available in the NCBI database. Performing the sequence alignment, there can be recognized three groups that posses no identities among the groups but only within them (Table 4). The approach based on finding of the most conserved sequences cannot be applied for all of them but only for an individual group. The above-mentioned fully mirror the actual molecular knowledge situation about the profilins. Profilins are a multigene family that were summarized by Jimenez-Lopez et al [7] as having the functions in actin dynamics, beside their allergenic potential, which was described for olive, birch, maize, hazel and timothy-grass.

Table 4. Profilin sequence of hazelnut stored in the NCBI database.

Group No.	Accession Code for Cor a 1	Type of Nucleic Acid	Sequences Similarity within the Group
1	AF323975.1 AF323974.1 AF323973.1 AF136945.1	mRNA	99%
2	Z72440.1 Z72439.1	DNA	75%
3	X71000.1 X70999.1 X70998.1 X70997.1	mRNA	96%–99%

Profilin is a class of plant pan-allergen, as the profilins from very different plant species are responsible for similar allergic reactions in sensitized patients. Moreover, 20% of all pollen allergic patients react to plant profilins and many of the pollen expressed proteins that bind on IgE in allergic patients have been found to be profilins [32]. Profilins are known to be expressed by up to ten different genes in plants [33]. Today, two main groups are recognized depending on their manner of expression. The first group is expressed constitutively and in all plant tissues and the second one is expressed only in the reproductive tissues [34]. The distribution of profilin in pollen depends on the stage of development and the specific isoforms expressed [35].

6. Spring Tree Pollinosis—Expression Analysis of Bet v1, Bet v2, Cor a 1 and Cor a 2 in Pollen Grains from DifferentIn Situ Conditions

Real-time PCR based expression analysis of food allergens per se is well established for almost all food allergens [36,37]. For pollen allergens, the situation regarding the expression of allergen genes in the pollen grains is very different. The application of real-time PCR in the analysis of the expression level of pollen allergens from differentin situ conditions is only in the experimental phase and no

normalized methods actually exist. Real-time PCR was reported as efficient in pollen monitoring by [38]. The authors have used nuclear ribosomal RNA genes as target for the quantization to improve conservation within species. The whole genome gene expression analysis was performed for olive pollen samples by [39], but the expression of specific pollen allergens directly in pollen was reported to be rare [40,41].

Real-time PCR has the potential to be used in routine comparative analysis of allergen expressionin situ and the subsequent health and land management can be applied, such as in the case of the results of different levels of birch Bet v1 expression. In the study of [40], different localities of Ukraine were chosen for the comparative study. Bet v1 expression in the pollen from different part of cities was from 0.77 to 2x higher as the pollen collected in the forest. Bet v1 expression in the pollen from village 0.55x higher when comparing to the forest sample. These findings have started the discussion about the presence of birch trees in the specific part of Kiyev (personal communication with Katerina Garkava—Institute of Ecological Safety, NAU in Kyiv). This is why setting up the validated methods will have very practical outcomes. In the case of the expression levels of CorA allergen in the *Corylus avellana*, the situation was reported to be similar. The level of CorA expression was within the range of 0.532 to 1.206x higher for the samples from urbanized area when compared to the sample from the village [41]. Very different data were obtained for the expression of hazelnut pollen profilin. The same samples as in the analysis of CorA allergen expression were used, but the results have shown a high variation in the abundance of profilin allergen transcripts. Expression levels were in the range of 2.957 up to the 52.936 higher when comparing samples from urbanized area to the sample from the village. In the downtown area, the profilin expression reached on average 21 times higher values and, in the sample from cement plant area, the expression is reported to be 52 times higher [41]. The interaction between air pollution and allergenic vegetation was reported previously [42] to be a consequence of the coverage of air pollution by micro-particulates. The results of the transcriptomic analysis [40,41] show that the effect of the higher allergenic potential of pollen grains in urbanized area has synergic background and the expression of allergens is higher there per se.

Real-time PCR is reported by many authors as a very efficient tool in many specific applications such as gene expression [43], identification and quantification of different pathogens [44] or authentication of food [45]. For the purposes of detection and quantification of airborne allergenic pollen taxa, real-time PCR was also applied. Longhi et al. [38] have reported the technique as a rapid, accurate, and automated for pollen grain quantitation and control.

Differences among levels of expression are well documented for many allergens and for different stages of the growth [46,47]. Wani et al. [48] reported that different expression levels of allergen were obtained for variable climatic conditions.

Today, different approaches can be applied for the development of real-time PCR protocol [49,50]. Regarding the pros of data mining, the first step of the transcriptomic analysis (Figure 3) in the case that mRNA sequences of allergens are known and stored in public databases is the sequences alignment. This is aimed to identify their conserved and variable parts. The most appropriate regions for primer designation can be mined by BLAST analysis [51] against the individual sequences. Using this approach, verification steps of the product specificity checking must be a part of the analysis work-flow. Here, HRM analysis or restriction cleavage can be chosen beside the melting analysis of the amplified target.

Another crucial step for the accurate quantification of analyzed transcripts is the identification of stable reference genes to normalize the target levels when using real-time PCR [52]. Those commonly used are housekeeping genes that are proven to be non-regulated. However, some studies reported high variation of tested housekeeping genes under different conditions and this is why no current universal reference gene exists [53], which is why the standard for using housekeeping genes is the approach where a set of reference genes (2–3 different genes) is validated [54].

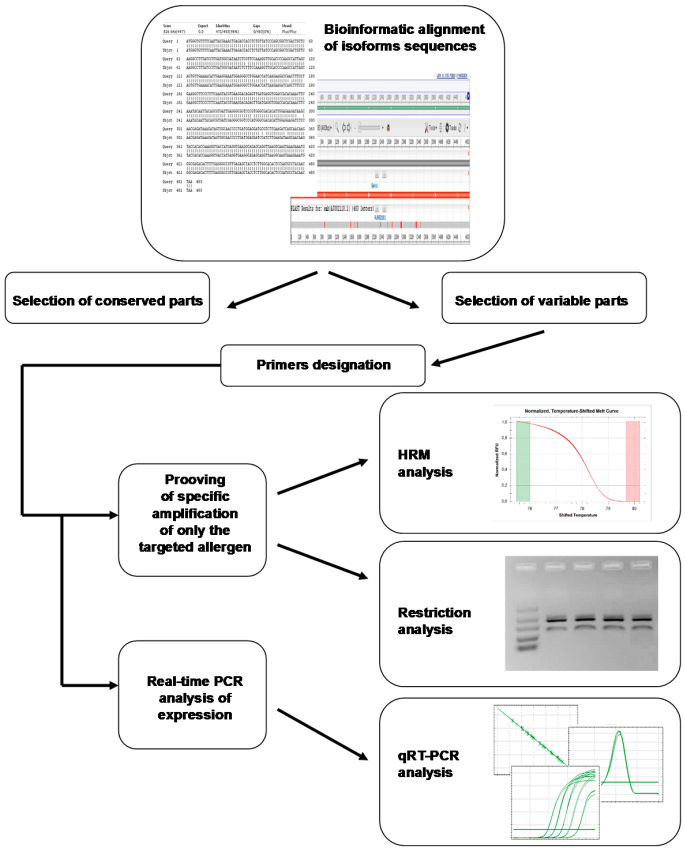

Figure 3. Real-time PCR analysis work-flow of allergen expression based on data mining.

Different housekeeping genes were reported as internal controls in real-time PCR analysis [55,56]. Cyclophylin, alpha-tubulin, and transcription factor CBF1 were tested as controls to normalize expression of Silver birch pollen Bet v1 allergen by [38]. Comparison of *Corylus avellana* pollen expression level using 18S rRNA as internal control was performed by [41]. HMG CoA reductase was validated for hazelnut pollen expression by [57].

7. Conclusions

The mechanism and regulation of allergens expression and the questions of regulatory processes is the research interest with a high impact for the future. All relevant findings will help to manage pollinosis that many people suffer with through the management of the biology of the allergens directly in the plants.

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List of Abbreviations

BLAST	Basic Local Alignment Search Tool
HRM	High Resolution Melting
qRT-PCR	quantitative Real Time Polymerase Chain Reaction
NCBI	National Center for Biotechnology Information
PCR	Polymerase Chain Reaction

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