

Utilization of specific primers in legume allergens based polymorphism screening

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Original article

Abstract

Different types of allergies became a part of life of many people around the world. The research activities connecting to allergens are actually not oriented only for protein and immunological interactions, but to the genomic and transcriptomic background of them, too. Analysis and description of genomic variability of allergens in plant food resources will help to manage the allergen based strategies in the future. Here, the bioinformatic approach was used to develop and validate the specific primers for genomic screening of polymorphism of profilins (Profilin Based Amplicon Polymorphism; PBAP) and vicilins (Vicilin Based Amplicon Polymorphism; VBAP) among the legumes. The alignment of existing public databases data for these allergens in the group of legumes was performed. Subsequently, specific primers were designed and their ability to generate polymorphic amplicons were tested for three legumes – bean, lentil and chickpeas. In all cases, amplicons were generated and polymorphism was detected in all three species for profilin as well as for vicilin.

Keywords

- legumes
- profilins
- vicilins
- specific primers
- polymorphism

Authors contributions

A – Conceptualization
B – Methodology
C – Validation
D – Investigation
E – Writing – original draft preparation
F – Writing, reviewing & editing

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Article info

Article history

- Received: 2021-09-30
- Accepted: 2021-11-22
- Published: 2021-11-22

Publisher

University of Applied Sciences in Tarnow
ul. Mickiewicza 8, 33-100 Tarnow, Poland

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Financing

This publication was supported by
the Operational Program Integrated
Infrastructure within the project: Demand-
driven Research for the Sustainable
and Innovative Food, Drive4SIFood
313011V336, cofinanced by the European
Regional Development Fund.

Conflict of interest

None declared.

Introduction

Food allergy has become a part of the life of many people around the world. The incidence of food allergic diseases is increasing. Although the prevalence is unknown, food allergy affects approximately 6-8% of children [1] and 1.5% - 2.5% of adults [2,3]. The principle of food allergy is an adverse reaction to an otherwise harmless food. The immune system responds to one or more proteins in food that are recognized as foreign, and the immune system triggers a response to neutralize them. The onset and extent of an allergic reaction depend on a person's genetics, the allergic condition, and the type and amount of allergen involved [4].

On a worldwide scale, legumes are important crop plants and represent more than 27% of the primary crop production, with grain legumes alone contributing 33% of the dietary protein nitrogen (N) needs of humans [5]. Legumes have a high nutritional value. Their seeds contain 20% to 30% protein [6]. Oil content is more variable from 1% (e.g. lentil) to 50% (e.g. peanut) [7]. However, they are responsible for a high incidence and severity of allergic reactions. Allergenic food proteins have been identified in nearly all of the most important legume crops, including peanut (*Arachis hypogaea*), soybean (*Glycine max*), lentil (*Lens culinaris*), common bean (*Phaseolus vulgaris*), mungbean (*Vigna radiata*), chickpea (*Cicer arietinum*), and pea (*Pisum sativum*) (Table 2). Unfortunately, there are no estimates of the prevalence of other legume allergies [8].

The prevalence of allergy to legume is variable and depends on the geographical location and the types of legumes. Higher legume consumption is responsible for increased sensitization [4]. The majority of legume allergens belong to mostly four protein families and superfamilies: cupins, prolamins, profilins, and pathogenesis-related proteins [9].

Profilins are small 14- to 17-kDa cytosolic proteins expressed in all eukaryotic cells [10]. The major role of profilin in plant cells is the rapid reorganization of microfilaments during processes like cytokinesis, cytoplasmic streaming, cell elongation, and growth of pollen tubes and root hairs [11-12]. Plant profilin was discovered by Valenta et al. [13] as an allergen source in birch pollen. Since then, the structure, physical and chemical properties, and functions of plant profilins have been widely and deeply studied and important progress has been made. Currently, about more than 400 profilin proteins from plants are available at NCBI (National Center for Biotechnology Information) gene database [14].

Vicilins belong to the superfamily of cupins, it is a functionally diverse superfamily of globular proteins with the so-called cupin domain, including seed storage

proteins. Vicilins (7 / 8S globulins) are trimeric proteins with a molecular weight from 150 to 190 kDa. The molecular weight of the subunit is approximately 40-80 kDa [15].

This study focuses on designation and validation of specific primers for the genomic screening of polymorphism of profilins (Profilin Based Amplicon Polymorphism; PBAB) and vicilins (Vicilin Based Amplicon Polymorphism; VBAB) among the legumes and tests the ability to generate a polymorphic amplicon for selected legumes.

Materials and Methods

Bioinformatics and in silico methods

Basic bioinformatic screening of available known nucleotide sequences was performed in NCBI and AllerBase databases. Subsequently, conserved parts of profilin and vicilin sequences were obtained by the BLAST algorithm in NCBI [16] and primers were designed by Primer3 software.

Plant material

The biological material of 3 species of legumes – bean, chickpea and lentil was used in the study. The plants for genomic analyzes of polymorphism profilins (Profilin Based Amplicon Polymorphism; PBAB) and vicilins (Vicilin Based Amplicon Polymorphism; VBAB) were grown in the *in vitro* conditions. The leaves were harvested from five weeks old plants and frozen immediately. The plants were stored at -50°C until further use.

DNA extraction and PCR reaction

Total genomic DNA was isolated by GeneJET™ Plant Genomic DNA Purification Mini Kit (Thermo Scientific) following the manufacturer's instructions. NanoPhotometer™ (IMPLEN) was used for quality and quantity checking of the extracted DNA.

The bioinformatics screening was performed to find the conserved parts of nucleotide sequences stored in the National Center for Biotechnology Information (NCBI) database for the genes of profilin and vicilin and for the designation of specific primers (Table 1). No specific data are stored for the profilins of legumes, that is why the *Rosaceae* species was used for finding the conserved parts of the profilins and the main comparison

was performed to the profiling sequences of apple. In the case of vicilin, the pea vicilin gene (accession code X14076.1) was used to be compared with other genes of legume vicilins stored in the NCBI database.

Table 1. The primers used in the study

Primer name	Nucleotide sequence (5' → 3')	
	Forward	Reverse
Profilin	ACCGGCCAAGA-TCTGGTTTT	AGGTAGTCTCC-CAACCTCTCC
Vicilin	AGGGATCTTT-ATTGTTGCCA	TCATTCTTT-GACCCACAAG

PCR reactions were performed in a 10 µl reaction mixture with EliZyme HS Robust MIX with 30 ng of DNA and 400 nM of specific primers. Amplification was performed in Biometra TProfessional Thermocycler under the following conditions: 95°C 5 min (95°C 45 s; 55°C 45 s; 72°C 35 s) 40x; 72°C 10 min.

Data analysis

PCR products separation was performed in 3% agarose gel in 1xTBE buffer stained with GelRed™ (Biotium) by agarose electrophoresis. The amplicons were visualized by BDAdigital system 30 (Analytik Jena). Profiles of PBAB and VBAB were analysed by software GelAnalyzer (www.gelanalyzer.com).

Results and discussion

Analysis of actual available data for profilin and vicilin sequences in selected legumes

A different situation exists for the knowledge about the individual allergens in plant species. In many cases, even the clinically most relevant allergenic molecules are not characterized on both levels, proteomic and genomic ones. For legumes, the most information is available for the model species and clinically the most relevant – peanut and soybean (Tables 2 and 3).

The symptoms of an allergic reaction associated with the consumption of legumes are similar to other food

allergens and are more or less similar for all legumes. People sensitive to these allergens can have mild to severe symptoms such as oral allergies, angioedema, hives, rhinitis, asthma, but also anaphylaxis, and in rare cases, death. Inhalation of vapor, powder, or flour from some legumes can create respiratory problems such as asthma and hypersensitivity pneumonitis [4]. In Spain, legumes are the fifth most common cause of food allergy, especially among children [17]. The estimated prevalence of lupine allergy in Europe and the Mediterranean ranges from 1.6% to 4.1% [18, 19]. The incidence and prevalence of peanut allergy are increasing every year. In 2018, approximately 2,2% of children and adolescents in the USA had peanut allergies [20]. Peanut allergy is one of the most common legume allergies [21]. Even a small amount of peanuts can set off an allergic reaction in sensitive individuals [22]. Adult patients with peanut allergy have been shown to have frequently clinically relevant sensitization to lupines, peas, and soybeans [23]. The best-identified allergens in peanuts include 7S globulin (vicilin) Ara h1 [24] and profilin Ara h5 [25]. Thermal processing of peanuts significantly reduced the allergenicity due to changes in the structure of the proteins when heated [26].

Among legumes, lentils seem to be one of the most common foods associated with allergic reactions in the Mediterranean and many Asian countries, especially among children [42]. Allergy signs and symptoms after ingestion of uncooked and cooked lentils can include oropharyngeal symptoms and acute urticaria, followed by anaphylaxis [43]. Several cases of anaphylaxis caused by inhaling vapors from cooking or eating lentils have rarely been described [44]. Thermal processing of lentils does not affect the change in immunoreactivity [33]. Allergenicity is significantly reduced for up to 30 minutes by autoclaving at 2.56 atm [45]. The main allergen in lentils is vicilin Len c 1 [33]. Len c 1 is 90% homologous to vicilin in pea Pis s 1 [37], and to more than 50% homologous to the major allergen in Ara h 1 peanuts [46] and the beta subunit of soy conglycinin [47]. Only Len c 1, Ara h 1 and Pis s 1 share common epitopes and therefore may elicit a cross-reaction [48].

The main allergen of lupine is the allergen Lup an 1 belonging to the family 7S vicilin like globulin [34] and Lup a vicilin [35]. Lupine allergy can result from a cross-reaction in people allergic to another member of the legume family, especially peanuts [18], in some cases resulting from primary sensitization [49]. Results [50] suggest that only autoclaving at 138 °C has a significant effect on the integrity and structure of allergens, thereby reducing the overall allergenicity.

The main pea allergens, which belongs to the 7S globulin family, are named Pis s 1 and Pis s 2 [37]. Pis s 1 has

Table 2. Well-known, identified, and characterized profilins and vicilins from different legumes

S. No	Allergen	Protein family	References
Peanut (<i>Arachis hypogea</i>)			
1	Ara h1	Vicilin (7S vicilin like globulin)	Burks et al. 1991 [24]
2	Ara h5	Profilin	Kleber-Janke et al. 2001 [25]
Soybean (<i>Glycine max</i>)			
1	Gly m3	Profilin	Ogawa et al. 2000 [27]
2	Gly m Bd28K	Cupin (7S vicilin like globulin)	Gonzalez et al. 1992 [28]
3	Gly m Bd 60 K	Cupin(7S vicilin like globulin)	Codina et al. 1999 [29]
4	Gly m 5	Cupin (7S globulin)	Holzhauser et al. 2009, Ogawa et al. 1995 [30, 31]
Mung beans (<i>Vigna radiata</i>)			
1	Vig r 5 (75)	Profilin	Mittag et al. 2005 [32]
Lentil (<i>Lens culinaris</i>)			
1	Len c1	Vicilin	Sanchez-Monge et al. 2000 [33]
Lupin (<i>Lupinus angustifolius</i>)			
1	Lup an1	Conglutin beta (7S seed storage globulin ,vicilin)	Goggin et al. 2008 [34]
2	Lup a vicilin	Cupin (7S globulin)	Peeters et al. 2007 [35]
White lupin (<i>Lupinus albus</i>)			
1	Lup a γ -conglutin (80)	Cupin (7S globulin)	Magni et al. 2005 [36]
2	Lup a vicilin (81)	Cupin (7S globulin)	Peeters et al. 2007 [35]
Pea (<i>Pisum sativum</i>)			
1	Pis s 1	Vicilin	Sanchez- Monge et al. 2004 [37]
2	Pis s 2	Convicilin	Sanchez- Monge et al. 2004 [37]
3	Pis s 5	Profilin	van Ree et al. 1992 [38]
Locust tree (<i>Robinia pseudoacacia</i>)			
1	Rob p 2 (82)	Profilins	Compés et al. 2006 [39]
Chickpea (<i>Cicer arietinum</i>)			
1	Cic a VCL	7S Vicilin-like Globulins	Bar-El Dadon et al. 2013 [40]
French bean (<i>Phaseolus vulgaris</i>)			
1	Pha v PSL	Vicilin	Rougé et al. 2011 [41]

a high degree of homology with vicilin Len c 1 (~90%) [41] and with Pis s 2 (~70%) [51]. Pis s 2, identified as convicillin, recognizes a higher percentage of individual sera from allergic patients and therefore appears to be the major allergen in peas [37].

Chickpea allergy is very specific, rare, and most often associated with lentil allergy [52]. A putative allergen in chickpeas is vicilin Cic a VCL and likely cross-reacts with the allergens of pea and lentils [40].

First, the *in silico* prediction was performed for the designed primers by using the available sequences of profilin and vicilin of legumes (Table 3).

Table 3. *In silico* prediction of generating amplicons in PCR based on actual data in NCBI for viciline

Species	Accession number of vicilin	F primer	R primer	Predicted polymorphic
Chickpea (<i>Cicer arietinum</i>)	X51908.1	Yes	Yes	Yes
French bean (<i>Phaseolus vulgaris</i>)	X03004.1	Yes	No	?
Lentil (<i>Lens culinaris</i>)	AJ551424.1	Yes	Yes	Yes

The sequences of the designed specific primers for vicilin were used to recognize the site of the corresponding template sequences actually available in the public databases. Each primer was examined using the BLAST algorithm for the sequences listed in Table 2. The specificity and efficiency of primers were verified further in PCR using the extracted DNA of selected legume species. Up to date, no profilin sequences of nucleic acids are available in the public databases, that is why this part of validation was performed directly by PCR. Based on the *in silico* primer binding, a polymorphism was predicted for species of *Cicer arietinum* and *Lens culinaris*. In the case of *Phaseolus vulgaris*, the prediction of polymorphism is questionable due to any founded binding site of the reverse primer.

Characteristic of amplified fragments

The transferability of designed specific primers for profiling and vicilin to PCR amplification was performed further. The analysis for three selected species – chickpea, bean and lentil, were performed.

Completely different amplicon profiles of PBAP were obtained for the analysed species (table 4) where the amplicons were generated within the range of 46 up to the 669 bp and all of the three species can be differentiated by this marker. Evaluation of genetic diversity and an understanding of the genetic relationships in the germplasm collection provide useful information to address breeding programmes and also confirm the usefulness of diverse germplasm in crop breeding programs. Chickpea has a narrow genetic background [53] and looking for desirable traits is fundamental for varietal improvement in different breeding strategies.

Table 4. Characteristics of obtained fragment length in selected legumes for profilin specific primers

Species	Length of amplified fragments by PBAP [bp]
Chickpea (<i>Cicer arietinum</i>)	117, 76, 46
French bean (<i>Phaseolus vulgaris</i>)	669, 530, 335, 135
Lentil (<i>Lens culinaris</i>)	192, 120, 89

The PBAP marker system seems to be generating a limited number of amplicons for legume species, as three different amplicons were obtained for chickpea and lentil and four amplicons were obtained (see Figure 1).

In the case of VBAP, similar results were obtained and different amplicon profiles were generated for the analysed species (Table 5), too. Here, the amplicons were obtained within the range of 83 up to 253 bp and all of the three species were differentiated by this marker.

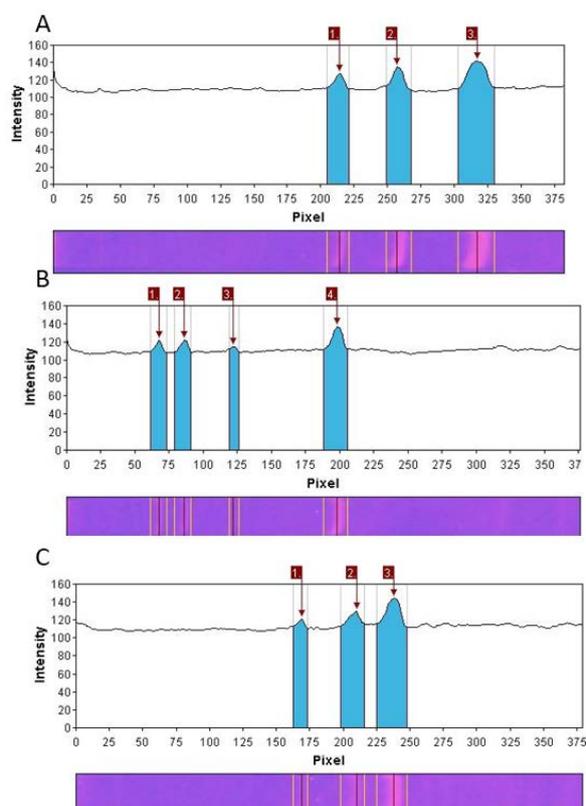


Figure 1. Fragment analysis of amplicons generated by PBAP for chickpea (A), bean (B) and lentil (C) evaluated with software GelAnalyzer

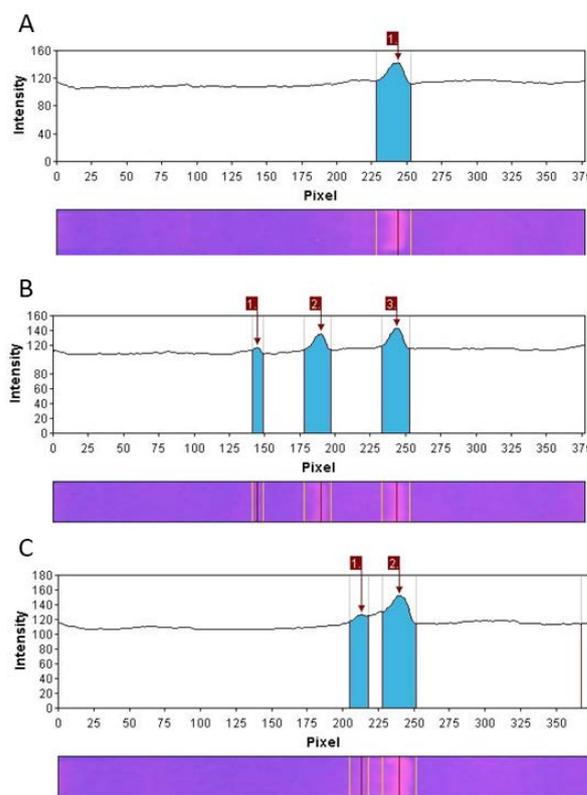


Figure 2. Fragment analysis of amplicons generated by VBAP for chickpea (A), bean (B) and lentil (C) evaluated with software GelAnalyzer

Table 5. Characteristics of obtained fragment length in selected legumes for vicilin specific primers

Species	Length of amplified fragments by VBAP [bp]
Chickpea (<i>Cicer arietinum</i>)	83
French bean (<i>Phaseolus vulgaris</i>)	253, 148, 83
Lentil (<i>Lens culinaris</i>)	112, 84

Here again, only a limited number of amplicons is generated by VBAP marker system for analysed legume species (Figure 2) and only one amplicon was amplified in the case of chickpea.

Both of the markers used in this study were proved to generate the polymorphism among legume species and we suppose they will provide valuable data for the analysis of the legume germplasm variability concerned specifically to coding regions of allergens.

Coding regions are normally used to describe the variability of legumes. DNA fingerprinting database was produced using three different PCR-based molecular marker systems (SSR, SCoT, and CDDP) for 48 chickpea genotypes collected from different sources. The results indicated that primers obtained from different regions of genomic DNA successfully amplified genotype DNAs. All of the three molecular marker sets were able to distinguish and identify each of the 48 chickpea genotypes. Salient features of fingerprinting database obtained using SSR, SCoT, and CDDP markers are given below [54].

Different tools were used to determine the genetic relationship between members of *Fabaceae* as a morphological marker and protein electrophoresis. But the relation at some plants needs additional techniques such as molecular markers like random amplified polymorphic DNA (RAPD) [55] and SCoT primers. SCoT markers provide easier development of species-specific primers than SSR [56], lower cost than AFLP [56] and higher reproducibility than RAPD [57]. In some cases, it is recommended that molecular markers such as SCoT should be employed to complement the findings of the protein electrophoresis study [55]. *Phaseolus vulgare*

was found alone within legume protein genetic diversity [58]. The relation between *Phaseolus vulgare* and *Cicer arietinum* was reported to be the same [59]. Also, the present results are supported by the rbcL data [60, 61]. Therefore, the results of the reported study show that SCoT markers are efficient in assessing the genetic diversity among *Fabaceae*.

Conclusions

Here, new types of DNA-based marker techniques for the analyse of *Fabaceae* members are reported – Profilin Based Amplicon Polymorphism (PBAP) and Vicilin Based Amplicon Polymorphism (VBAP). Proteins homologous to main allergens are abundant in the plant kingdom, and the homology of their genes provides a sufficient base for the functionality of DNA markers based on their sequences. The functionality of the PBAP and VBAP were proved for different legume species and was resulted in universal marker systems.

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