JMBFS

Journal of Microbiology, Biotechnology and Food Sciences

International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

VARIABILITY OF AMPLIFIED PROFILES GENERATED BY BBAP IN *AVENA SATIVA* L.

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https://doi.org/10.55251/jmbfs.9545

ARTICLE INFO

Received 17. 10. 2022
Revised 2. 2. 2023
Accepted 20. 2. 2023
Published 3. 4. 2023

Regular article

OPEN ACCESS

ABSTRACT

Avena sativa L. is a very common specie for human consumption that provide different health benefits. As a dual purpose crop, oat provide a source of protein, fiber and minerals. Here, homologs of Bet v 1 allergen genes were used as DNA markers to analyse their utilization for genomic variability of oat varieties. The presence of Bet v 1 homologues in seven oat varieties were examined and their length polymorphism was analysed. Bet v 1 based amplified profile – BBAP- was used to obtain the polymorphic profiles for the used oat varieties by a combination of specific and degenerated primer pair. This method was reported to be universal in plants for screening of variability of coding regions. Using the primer pair Bet v 1 forward and degenerated Bet v 1 reverse 49 amplicons were amplified in a total. The length of the amplicons varied from 265 base pairs to 1929 base pairs. All analysed oat varieties generated unique amplicons, but there was not a size of amplicon that all cultivars shared. Constructed dendrogram based on hierarchical cluster analysis using UPGMA algorithm grouped the analysed varieties genotypes into three main clusters – Black Beauty and Zvolen varieties, Valentín and Earl and Vendelín varieties and Floron and Dagles varieties. BBAP technique provide a high polymorphism in the Bet v 1 isoforms present in oat and all of the analysed varieties were distinguished what showed the utility of this marker technique for analysis of genetic diversity and genotype identification of oat varieties.

Keywords: *Avena sativa* L., allergens, BBAP, DNA markers, polymorphism

INTRODUCTION

Cultivated varieties of *Avena sativa* L. are hexaploid cereals belonging to the genus *Avena* L. Thank to their agronomic adaptation, this specie is planted worldwide in almost all agricultural environments. Actually, oats have been receiving increasing interest for the purposes of human nutrition, mainly because it could be suitable for consumptions by celiac patients **(Gálová *et. al.*, 2012),** but the other group of health threating potential of oat is connected to allergies. Allergic diseases are complex and extensive in terms of organs affected and severity, from highly prevalent allergic rhinitis to life-threatening anaphylaxis **(Pomés *et al.*, 2018)**. Allergic reactions require complex interactions between the protein and the immune system and are therefore difficult to predict. Nevertheless, it is clear that some proteins are inherently more allergenic than others **(Huby *et al.*, 2000)**. Due to the general increase in allergic sensitization, the prevalence of hypersensitivity reactions to multiple foods that share homologous proteins has become a significant clinical problem. Conservation of these proteins across biological tissues affects cross-reactivity. Originally, isoallergens were defined as different molecular forms of the same allergen that share intense cross-reactivity (IgE). The revised nomenclature states that isoforms are allergens originating from the same species with similar molecular structure and biological function, and are sequentially identical to at least 67% **(Lockley, 1998; Pomes *et al.*, 2018; Larsen *et al.*, 1996)**.

One of the most studied allergens are pathogenesis-related (PR) proteins. PRs represent a heterogeneous group of 14 plant protein families. Many plant food allergens are homologous to pathogen related proteins **(Breiteneder and Radauer, 2004; Carr and Klessig, 1989)**. The current classification divides the PR proteins into 17 classes, PR-1–PR-17 **(Fernandez *et al.*, 2013)**. The classification as PR-proteins is based on the induced expression in response to pathogen infections by viruses, bacteria or fungi, to wounding or to abiotic stress **(Schenk *et al.*, 2009)**. Due to its natural content in the plant cells, they are in the focus of the interest as coding DNA markers, too (**Žiarovská and Zeleňáková, 2018)**. Allergens known as PRs include chitinases (PR-3 family) found in avocado, banana, and chestnut; proteins that have antifungal character such as the thaumatin-like proteins (PR-5) found in cherry and apple; proteins homologous to the major birch pollen allergen Bet v 1 (PR-10) from vegetables and fruits; and lipid transfer proteins (PR-14) mostly from fruit and cereals **(Breiteneder and Ebner, 2000)**.

One of these classes represent PR 10 proteins. There is no evidence yet for a specific role for PR-10 proteins in the plant cell system, but their conserved sequence motifs and the fact that they are widespread throughout the plant kingdom suggest a general and important function. They are expressed when plants encounter abiotic and biotic stress, on that basis they probably have a protective role. However, it is important to highlight that some PR-10 members are also constitutively expressed, suggesting a more general biological role in plant development. PR-10 proteins are encoded by multigene families, this is probably the basis of the multifunctional aspect of these proteins **(Fernandez *et al.*, 2013)**. Developmentally regulated isoforms that are expressed at high concentrations in pollen, seeds, fruits were identified as allergens cross-reacting with Bet v 1. Phylogenetic analyses of PR-10 isoforms revealed that multiple sequences from each plant family formed a monophyletic group and sequences from each family were generally more similar to each other than to sequences from other families, suggesting that the genes of the multigene family have undergone strong coordinated evolution. In recent years, a superfamily of Bet v1-related proteins has been established that extends beyond the PR-10 family **(Radauer and Breiteneder, 2007)**.

According to **Ricci *et al.* (2005)** grass pollinosis is frequently associated with polysensitization to other pollen and food allergens. Bet v 1 specific IgE are significantly higher in these patients than in patients with grass monosensitization, and this sensitization may be considered a possible risk factor to evolve later into food allergy. In spite of the small clinical relevance of Bet v 1 grass pollinosis, the coding parts of Bet v 1 provide a source of DNA based variability among them. When we take into account that cereal grains (e.g., wheat, rye, barley, and oats) share homologous proteins with grass pollens and with each other **(Sicherer, 2001)**, we can expect that Bet v 1 gene homologs are present in cereals in such ubiquitous counts that the homology of these sequences should be analysed by the polymorphic marker technique – Bet v 1 based amplification polymorphism (BBAP). The BBAP method which was designed by **Žiarovská and Zeleňáková (2016)** and its universality was proved by **Urbanová and Žiarovská (2021)**, is a polymorphism generating PCR based technique that analyse the amplicon length variability of amplified regions of Bet v 1 homologues (Figure 1). This method was successfully applied in different plant species to analyse the genomic variability such as *Malus domestica* Borkh. **(Speváková *et al.*, 2021)** or vegetable species **(Urbanová and Žiarovská, 2021)**. Here, the first application for the cereal species is reported.

Figure 1 Schematic representation of sources for BBAP generated length/amplicon polymorphism of homologs of Bet v 1 (NCBI accession P15494). Mutational variability was reported for sites marked by red asterisks **(Spangford *et al*., 2003; Urbanová and Žiarovská, 2021)**. Red box marks the epitope of Bet v 1 **(Brier *et al*., 2018)** that is a part of forward primer. Red arrows mark the positions of primers used in the BBAP.

Different DNA markers were used previously to analyse the diversity or identification of individual oat verieties, such as highly informative SSRs loci **(Pipan *et al*., 2021)**, SNP markers **(Tanhuanpää *et al*., 2006)**, ISSR **(Cieplak *et al*., 2021)** or SCoT loci **(Balážová *et al*., 2017)**. Differen techniques used for assessment of the genetic diversity of cultivated oat varieties provide a knowledge base for breeding programs. Basic data from DNA based markers allow the very precise determination of genetic differentiation level of cultivated oat varieties and their genetic distinctiveness.

The objective of this study was to determine the BBAP profiles for *Avena sativa* L. in selected varieties and to analyse the applicability of this technique for the purposes of description of genetic variability in this specie.

MATERIAL AND METHODS

Biological material

In the study grains of 7 different varieties of oats (*Avena sativa* L.) were used – Zvolen, Valentín, Dagles, Vendelín, Earl, Black Beauty, Floron. The surface of all the grains was sterilized by NaOH and kept frozen at - 20 ℃ until processing into samples. The grains were homogenized by grinder the day of DNA extraction.

Genomic DNA extraction

EliGene® Plant DNA Isolation Kit (ELISABETH PHARMACON, Brno, Czech Republic) was used to extract DNA from the samples and the supplier's instructions were followed. The quality and quantity of the extracted DNA was assessed spectrophotometrically by Nanophotometer P360 (IMPLEN, GmBH, Munchen, Germany). Control PCR reaction with ITS (internal transcribed spacer) primers was performed to assure of the usability of DNA in PCR reactions. In the control PCR, ITS2 (forward) and ITS 4 (reverse) primers and ITS 5 (forward) and ITS 1 (reverse) primers were used according to **White et al. (1990)**. The PCR conditions were as follows: initial denaturation at 95 ℃, followed by 35 cycles at 95 ℃ for 30 s, 55 ℃ for 30 s and 72 ℃ for 45 s, final extension was held for 15 min at 72 ℃.

PCR reactions and data analysis

The primers were chosen for the study according to **Žiarovská and Zeleňáková (2018)** and **Urbanová and Žiarovská (2021)**. The reverse primer (R) for Bet v 1 is degenerated on the 12. and 14. position. In the 12. position there can be guanine or cytosine; and in the 14. position can be thymine or guanine. The forward primer is not degenerated.

The PCR reactions were performed on TProfessional Basic gradient XL (BIOMETRA, Jena, Germany) thermocycler. All PCR reactions were performed in 10 µl volume, primers were added in 400 nM concentrations, 2 µl of DNA at concentration 20 ng. µl-1 were added with MasterMix Robust HS Elizyme (ELISABETH PHARMACON, Brno, Czech Republic). The thermal profile followed the steps: initial denaturation at 95 ℃ followed by 40 cycles of denaturation at 95 ℃ for 45 s, annealing primers at 54 ℃ for 45 s and elongation at 72 ℃ for 35 s; final elongation was held at 72 ℃ for 10 min according to **Urbanová and Žiarovská (2021)**. The PCR fragments were separated on 2 % agarose gels stained with GelRed® Nucleic Acid Gel Stain (BIOTIUM, Fremont, USA) and visualized by UV- transilluminator- BDAdigital system 30 (Analytik Jena, Jena, Germany). According to the amplicons binary matrixes were created with the help of free online software Gelanalyzer (www.gelanalyzer.com). Distance matrixes based on Dice coefficient were created than UPGMA dendrograms were constructed with free online software (http://genomes.urv.cat/UPGMA/).

RESULTS AND DISCUSSION

Genetic markers are not influenced by environment, so they are widely used to determine genetic variability among varieties in plants. There are many genetic marker systems that are used today. Genetic variability detected by molecular markers is useful in understanding of genome dynamics and in marker assisted selection breeding **(Varshney, 2010)**. In general, marker polymorphisms are a variety of point mutations arising from single nucleotide substitutions, rearrangements involving insertions or deletions, duplication of DNA sections, translocations, and inversions, as well as DNA replication errors that are tandemly repeated **(Amiteye, 2021)**. For the first time to our knowledge Bet v 1 generated homologous were used as DNA based polymorphic marker for *Avena sativa* L. In other plant species **Urbanová and Žiarovská (2021)** showed that the coding region of Bet v 1 can be used as DNA based molecular marker for different vegetable species. In the study of **Speváková et al. (2021)** the BBAP method was used for 10 apple varieties, they observed low level of polymorphism in amplification patterns, they concluded that isoforms within the selected apple varieties were genetically stable.

The reverse degenerate primer proved to be the most suitable primer for polymorphism evaluation, 49 amplicons were amplified in total. The length of the amplicons varied from 265 bp (base pair) to 1929 bp. Zvolen generated 10 amplicons, from size 269 bp to 1497 bp. Cultivar Valentín generated 7 amplicons from 280 bp to 1929 bp. Dagles and Earl both generated only 4 amplicons, in term of size from 280 bp to 366 bp and 270 bp to 371 bp. Vendelín generated 7 amplicons from 277 bp to 368 bp. Black beauty generated 8 amplicons (from 268 bp to 1929 bp) and Floron generated 7 amplicons (from size 265 bp to 1812 bp). All cultivars generated unique amplicons, there was not a size of amplicon that all cultivars shared. Valentín and Black Beauty generated 1929 bp long unique amplicon.

Zvolen, Valentín and Dalqes generated amplicons at size 289 bp, amplicon at size 324 bp was generated by Zvolen, Valentín, Dalqes and Vendelín. Vendelín, Earl and Floron generated amplicons at 282 bp and 371 bp. Distance matrix based on Dice coefficient we can see in Table 1. Based on this distance matrix an UPGMA dendrogram was constructed which we can see in Figure 1. Two main groups were formed, the first one included Zvolen, Valentín, Dalqes and Black beauty. The second one consisted of Vendelín, Earl and Floron. Valentín and Earl and Valentín and Dagles are less dissimilar than the other cultivars based on length polymorphism on the Bet v 1 gene present in the genome of these cultivars.

Table 1 Distance matrix based on Dice coefficient for the polymorphism of Bet v 1 gene in oat varieties

	Zvolen	Valentín	Dagles	Vendelín	Earl	Black Beauty	Floron
Zvolen	0	0.647	0.714	0.778	1.000	1.000	1.000
Valentín		0	0.455	0.867	1.000	0.600	0.867
Dagles			0	0.833	1.000	0.667	1.000
Vendelín				0	0.500	0.875	0.750
Earl					0	0.833	0.667
Black Beauty						0	0.875
Floron							0

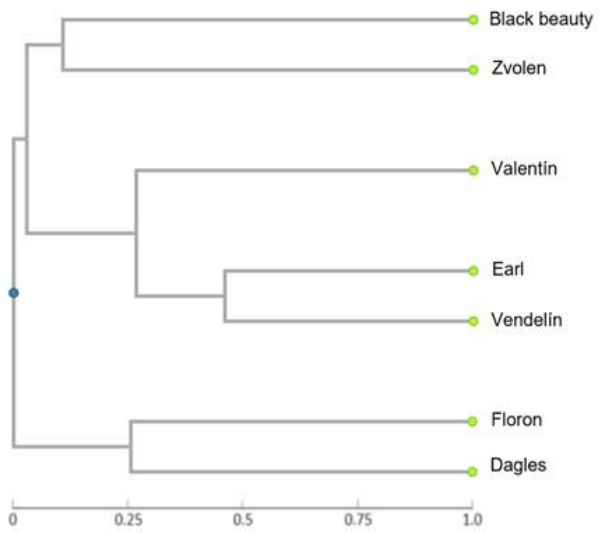

Figure 2 Dissimilarity dendrogram for length polymorphism of Bet v 1 homology genes in 7 *Avena sativa* L. varieties

The Cophenetic Correlation Coefficient (CP) = 0.859 presents that the clustering was fit. The most similar BBAP profiles that represent the sequence homology background of Bet v 1 genes in analysed oat varieties were obtained for varieties Earl and Vendelín together with Dagles and Valentín (Figure 3). When compared the effectivity individual variants of degenerated BBAP reverse primer and their amplification results, not all of them were suitable for polymorphism study of oat varietes. The reverse primers R1 and R2 **Urbanová and Žiarovská (2021)** with the combination of forward primer produced amplicon around the size of 450 bp in all cultivars. R2 produced more polymorphic products than R1, but not in all cultivars (Figure 4), Valentín produced only 3 products. Primer R3 produced monomorphic product at size 50 bp in all varieties, varieties Zvolen and Dagles had more polymorphic product than the other varieties. R 4 primer produced polymorphic amplicons in all varieties expect Dagles and Vendelín, which ones only had one amplicon.

Regarding the genetic polymorphism between oat cultivars, several studies have been carried out to this date with many DNA marker systems. **Pal *et al.* (2002)** studied 36 oat varieties, the frequency of polymorphic products ranged from 0.32 to 0.76 for ISSR markers (inter simple sequence repeats) and from 0.29 to 0.61 for SCoT (start codon targeted) markers. **Ruwali *et al.* (2013)** describe genetic variability among 15 oat genotypes including forage and dual-purpose oat varieties from different geographic regions. The results indicated a considerably high genetic diversity among the oat genotypes studied.

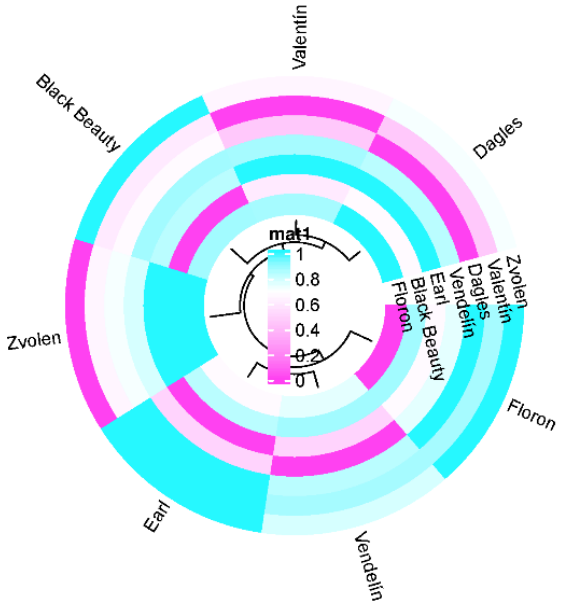

Figure 3 Heatmap of calculated genetic similarity of BBAP profiles of analysed *Avena sativa* L. varieties

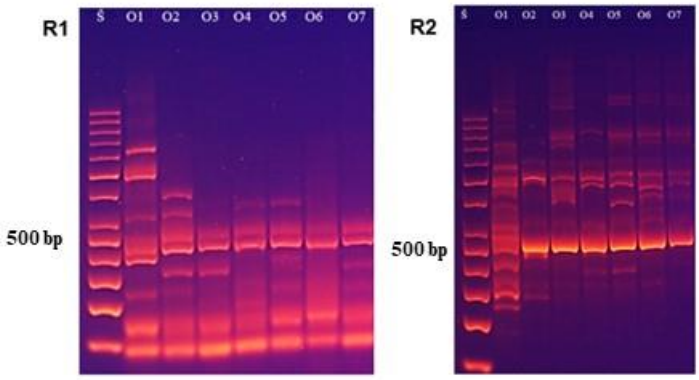

Figure 4 Obtained BBAP profiles of analysed *Avena sativa* L. varieties for R1 and R2 variants of degenerated reverse primers used in the PCRs. Š – length marker 100bp, O1 – Zvolen, O2 – Valentín, O3 – Dagles, O4 – Vendelín, O5 – Earl, O6 – Black Beauty, O7 – Floron.

Up to know, different studies of oat genomic variability were performed. **Boczkowska *et al.* (2013)** reported in an extensive study that using four AFLP primer pairs yielded a total of 231 fragments, of which 62.77% were polymorphic. The average number of fragments per primer pair was 57.8. During PCR reactions with eight ISSR primers, 509 fragments were amplified, of which 377 (74.1%) were polymorphic. The average number of fragments per primer was 63.6 and ranged from 49 (ISSR 5) to 85 (ISSR 3). The percentage of polymorphism ranged from 64.4% (ISSR 6) to 84.3% (ISSR 7). Only one RAPD marker was successfully used in the study. It generated 30 fragments, of which 80.0% were polymorphic. **Balážová *et al.* (2017)** studied 8 oat genotypes based on 18 SCoT markers, with 18 primers produced polymorphic data. Another study of oats varieties based on the SCoT markers of combination of 20 common oats and 2 naked oats by 7 SCoT primers reported 40 amplified fragments where 26 were polymorphic **(Chňapek *et al.*, 2022)**. **Cieplak *et al.* (2021)** also showed that SCoT and ISSR marker systems are suitable for genetic diversity studies for oats, their results showed that oats currently grown in Central Europe show high genetic similarity. The results of **Boczkowska and Tarczyk (2013)** showed similar high genetic similarity between oat landraces in Poland using ISSR markers. ISSR markers were also used by **Sharma *et al.* (2016)** to investigate polymorphism among oat mutant populations. **Sood *et al.* (2014)** write that, according to their findings, SSR (Simple Sequence Repeats) markers showed a higher level of polymorphism than RAPD markers within the genus *Avena*. Their findings also showed low diversity among commercial oat cultivars. **Montilla–Bascón *et al.* (2013)** studied the diversity between oat cultivars and oat landraces with SSR primers and EST (Expressed Sequence Tags), according to their findings, oat cultivars showed a lower level of diversity than landraces. Studies mentioned above show DNA marker systems used widely in the study of plant genomes, however the search for new DNA markers is ongoing, we present a potential new technique for oats to evaluate DNA polymorphism.

CONCLUSION

Bet v 1 homologues were detected in seven oat varieties. Degenerated reverse primer, designed for the conserved region of Bet v 1 gene detected high variability in the studied oat varieties. In the UPGMA dendrogram, 7 analysed oat varieties were divided into three main clusters, what points a good potential of these technique for oat genomic variability studies. It was possible to distinguish all analyzed varieties of oat in the constructed dendrogram based on BBAP markers. BBAP markers were revealed to be suitable for application in the studies of *Avena sativa*, L.

Acknowledgements: 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 (50%) and KEGA 020SPU-4/2021 Innovation of the methodological background and content of profile food and gastronomic subjects with a focus on increasing the competitiveness of graduates (50%).

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