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FAGOPYRUM SPP FINGERPRINT VARIABILITY USING THE ALLERGEN CODING SEQUENCES

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ABSTRACT

Buckwheat is one of the plant that possess a high nutrient benefits when used as human food. Up to now, different DNA markers were used to describe intra- and interspecie variability of this plants. Here, BBAP (Bet v 1 amplified polymorphism), PBAP (Profilin based amplified polymorphism) and VBAP (Vicilin based amplified polymor-phism) methods were applied to analyse their ability to distinguish the varieties and genotypes of *Fagopyrum esculentum* Moench and *Fagopyrum tataricum* Gaertn. For all of them, polymorphic profiles were obtained with diversity index ranged from 0.87 u to the 0.91. The ability of the primers to distinguish among the analysed varieties and genotypes was highest for BBAP. None of the applied marker techniques was specific for analysed buckwheat species.

Keywords: buckwheat, polymorphism, allergen based markers

INTRODUCTION

Buckwheat species belong to the Polygonaceae group of weeds, and its fruit can be used as food. It has become popular in many countries as a health food during the last decades (**Li *et al*., 2001; Gabrovska *et al*., 2002; Sofi et al., 2023**). Fagopyrum genus belongs to minor food crops and pseudocereals and is one of consumed plant food in arid and cold regions (**Bashir *et al*., 2021; Joshi *et al*., 2023**). Cultivation of common buckwheat (*Fagopyrum esculentum* Moench) and Tartary buckwheat (*Fagopyrum tataricum* Gaertn.) and is widespread mainly in the northern hemisphere such in Asia, Europe, and North America but actually China is one of the world's leading producers. Buckwheat most probably originated from southwest China where it is still cultivated in mountainous areas (**Luthar *et al*., 2021; Song *et al*., 2022**). A buckwheat grain is rich in carbohydrates, fiber, lipids, minerals, but also antioxidants (**Tang *et al*., 2016**). Rutin stands out as the most important flavonoid present in buckwheat - the highest concentration has been determined in the dry matter of Tartary buckwheat (**Rana *et al*., 2016**). Buckwheat flour does not contain gluten what make it suitable for people suffering from celiac disease (**Bashir *et al*., 2021**).
Beside the content of beneficiary nutrients, buckwheat genetic resources are characterized for their genetic variability, where different DNA based markers were used. The AFLP (Amplified fragment Length Polymorphism) technique was used to develop the first chromosome maps
of common buckwheat using genome-wide markers (**Yasui *et al*. 2004**). Microsatellite based SSR (Simple Sequence Repeats) technique was widely used in buckwheat germplasm characterization). The first SSR marker system in common buckwheat was reported by **Iwata *et al*. (2005)**. Fifty-four marker sets were developed, and a common buckwheat linkage map consisting of SSR and AFLP markers were constructed
(**Konishi and Ohnishi, 2006**). SSR markers have also been used to demonstrate that there is a low level of gene flow between wild and cultivated common buckwheat (**Konishi and Ohnishi 2007**). An assessment of the genetic diversity of 63 buckwheat genotypes was conducted using the SSR and ISSR markers by **Sabreena *et al*. (2021)**. The selected SSR markers was concluded as to be a suitable tool for the detection of polymorphism at the DNA level, which enabled an effective differentiation and characterization of the varieties of buckwheat (**Balážová *et al*., 2024**). DNA markers based on quantitative trait loci analyses were used in photoperiod sensitivity research using markers for expressed sequence tags (**Hara *et al*., 2011**). The same genotypes of common buckwheat and Tartary buckwheat as used in this study, were analysed for their SCoT (Start-codon target) polymorphism by **Mikolášová *et al*. (2022)** with a result that the average value of PIC for used SCoT markers was higher than 0.8 in 90 % of used SCoT markers what means sufficient polymorphism detected. Techniques to analyse DNA polymorphism based on RNA sequencing, using next-generation sequencing have been developed with a purposed to provide a technique that will be able to distinguished buckwheat varieties (**Moragues *et al*., 2010**).
Despite its many benefits, buckwheat seeds also contain potent proanaphylactic allergens (**Park *et al*., 2016**). Buckwheat allergy is an IgE-mediated allergy, sometimes causing severe allergic reactions (**Wieslander, 1996; Wieslander and Norbäck, 2001**). The buckwheat storage proteins, such as 13 S globulin and 2S albumin, have been reported as the major allergens of buckwheat (**Monshi et *al*., 2022**). Allergic reactions can occur when eating buckwheat food products, in the work environment or when sleeping on pillows containing buckwheat husks (**Norbäck and Wieslander, 2021**). There is reported cross-reactivity between buckwheat and other food allergens. Clinically relevant cross-reactivity have been found for latex (**De Maat-Bleeker and Stapel, 1998; Nawa *et al*., 2000**), poppy seed (**Oppel *et al*., 2006; Varga *et al*., 2011**), coconut (**Cifuentes *et al*., 2015**), quinoa (**El-Qutob *et al*., 2014**), and peanut (**Kobayashi *et al*., 2012**). Reported cross-reactivity of buckwheat is important for the possibility of utilization of DNA based markers specific for plant allergen homologs in its studies. DNA markers could be developed not only based on completed sequences information, but an *in silico* prediction of DNA markers was reported to be efficient (**Žiarovská and Zeleňáková, 2019**). *In silico* approach is used to find conserved parts in the genomic sequences of allergens in plant species and based on its alignment, specific or degenerate primers are designed (**Hovaňáková *et al*., 2024**). Plant allergens share a high degree of sequence homology for proteins, as well as for genomic sequences (**Jenkins et *al*., 2005; Nedyalkova et *al*., 2023**), what allow to develop efficient DNA markers. Here, an abundant plant allergens PR-10 and profilin and legume specific allergen vicilin were used as DNA markers. All of them were reported to be applicaple in fingerpring of plants. Previously, BBAP (Bet v 1 based amplified polymorphism) was successfully applied as a fingerprint method for various of plant species, as the sequences of Bet v 1 – main pollen allergen of birch – is highly conserved in its epitops in plants (**Urbanová and Žiarovská, 2021; Žiarovská and Urbanová, 2022**). In studies of soybean and groundnut, profiling and vicilin based DNA markers provided polymorphic profiles where a total of 16 different amplicons were obtained for profilin and 17 different amplicons were obtained for vicilin in a set of 30 different soybeans varieties and the PBAP (profilin based amplified polymorphism) technique was able to distinguish all of the analyzed varieties (**Kováčik *et al*., 2024**). In the case of groundnut, both of the techniques distinguished 31 analyzed accessions and VBAP (vicilin based amplicon polymorphism) provided polymorphism of 100 % (**Žiarovská *et al*., 2023**).
The aim of this study was to analyze the presence and level of polymorphism of BBAP, PBAP and VBAP for common buckwheat and Tartary buckwheat and compare the effectiveness of used marker techniques.

MATERIAL AND METHODS

Biological material

Totally, twenty-one different *Fagopyrum esculentum* genotypes and varieties were analysed in this study. The individual accessions were delivered from a collection of the Gene Bank of the Research Institute of Plant Production (Piešťany, Slovak Republic) and from collection of Czech Gene Bank (Prague, Czech Republic). They were planted in pods.

DNA extraction

g DNA was isolated by a commertial kit - GeneJET™Plant Genomic DNA Purification Mini Kit (Thermo Scientific, Waltham, MA, USA) where the manufacturer instruction was without any changes. Parameters of purity of obtained gDNA were spectrophotometrically analysed using the Nano-Photometer™ (Implen) and the functionality of isolated gDNA was performed in PCR tests using the universal ITS primers (**White *et al*., 1990**).

Polymorphism analysis

In the techniques of individual allergen homologs that were utilized here, primers were designed by the *in-silico* approach where the conserved sequences of allergens were used (**Žiarovská and Zeleňáková, 2019; Klongová *et al*., 2021; Kováčik *et al*., 2024**). Degenerated primer pair was designed in the case of Bet v 1 and profilin techniques and nondegeneraded in the case of vicilin technique. Robust polymerase (Elizabet Pharmacon, Czech Republic) was used with the concentration of 600 nmol of each used primer. PCR time-temperature profile was with following parameters: 95 ◦C for 5 minutes followed by 40 cycles of - (95 ◦C - 45 s; 55 ◦C - 45 s; 72 ◦C - 35 s) plus final elongation at 72 ◦C for 10 minutes. Generated amplicons were separated in 2% agarose gel stained by GelRed® (Biotium, San Francisco, CA, USA) and subsequently prepared as binary matrices. Dendrograms were prepared by the UPGMA analysis using the Jaccard index of genetic dissimilarity (**Jaccard, 1908**). Heatmaps were prepared by P-heatmap package in RStudio (**Kolde, 2019**).

RESULTS AND DISCUSSION

BBAP polymorphism

A total of 100 amplicons were obtained by Bet v 1 degenetated primer in the analysed buckwheat varieties/genotypes. Amplified fragments were polymorphic, but two groups possess in group the same and betweed groups different fingerprint profiles (figure 1). Dendrogram that was generated from obtained binary matrix has the value of cophenetic correlation coefficient 0.84. Based on Jaccard coefficients, analysed accessions are of a middle similarity (figure 2) and varieties Winsor Royal and Tonho Zairai were the most distinctive on their BBAP fingerprints.

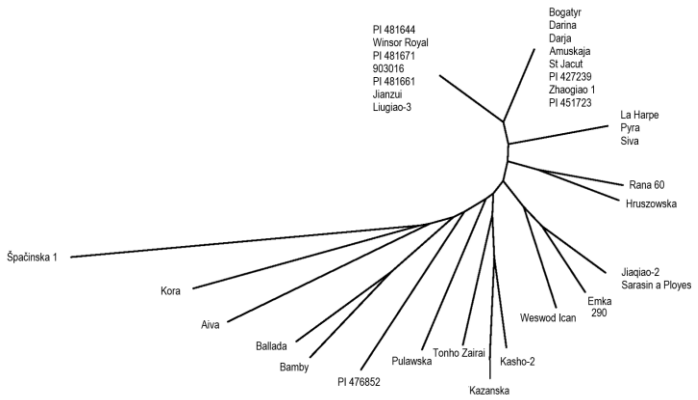

Figure 1 Dendrogram of obtained BBAP profiles for analysed buckwheat varieties/genotypes.

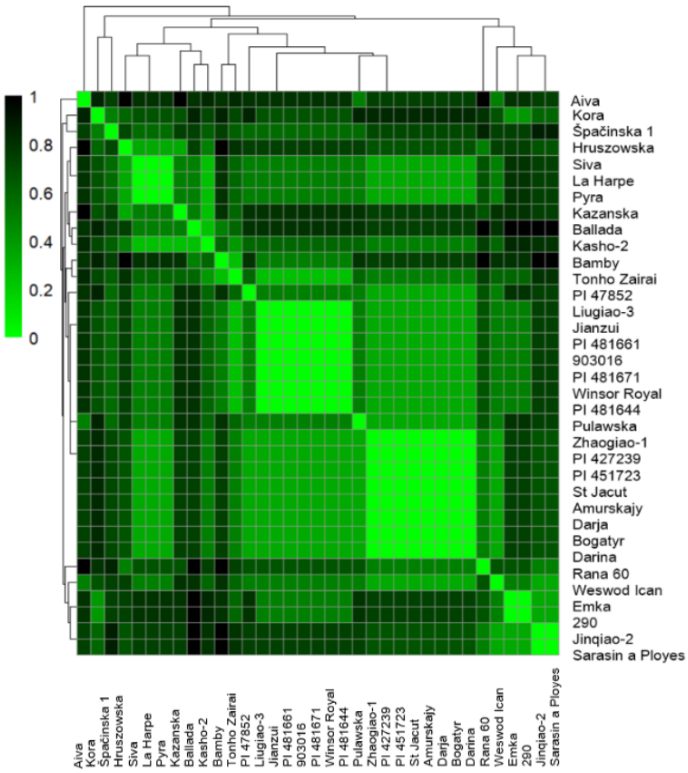

Figure 2 Heatmap of distance matrix of obtained BBAP profiles for analysed buckwheat varieties/genotypes.

PBAP polymorphism

A total of 88 amplicons were obtained by profilin degenetated primer in the analysed buckwheat varieties/genotypes. Amplified fragments were polymorphic, but for profiling, six groups of genotypes were obtained, that have the same profile inside the group, but different to other profiles (figure 3). Dendrogram that was generated from obtained binary matrix has the value of cophenetic correlation coefficient 0.75. Based on Jaccard coefficients, analysed accessions have a higher range of similarity among themselves (figure 4) and varieties Kora and Kasho-2; Pulawska and La Harpe and Špačinska 1 and Hruszowska were the most distinctive on their PBAP fingerprints.

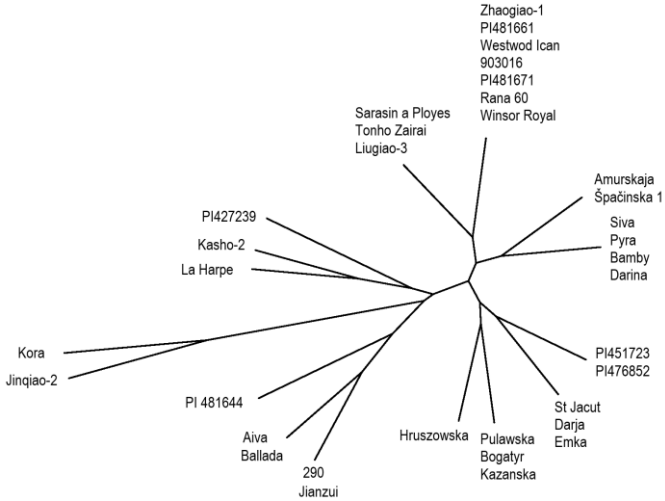

Figure 3 Dendrogram of obtained PBAP profiles for analysed buckwheat varieties/genotypes.

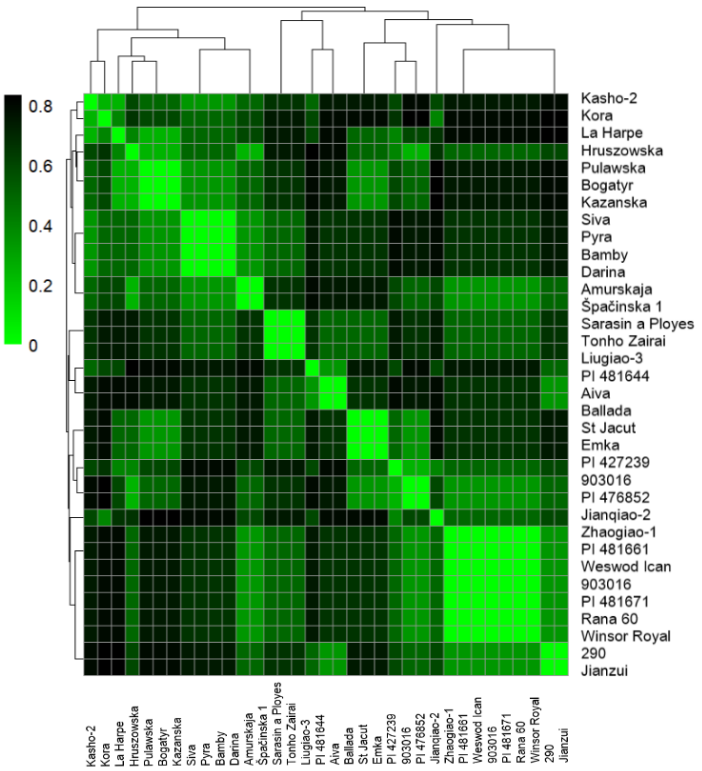

Figure 4 Heatmap of distance matrix of obtained PBAP profiles for analysed buckwheat varieties/genotypes.

VBAP polymorphism

A total of 86 amplicons were obtained by vicilin primer in the analysed buckwheat varieties/genotypes. Amplified fragments were polymorphic, but here, a large group of varieties/genotypes was obtained, where only two amplicons were amplified (figure 5). Dendrogram that was generated from obtained binary matrix has the value of cophenetic correlation coefficient 0.90. Based on Jaccard coefficients, analysed accessions have the highest range of distance among themselves (figure 6).

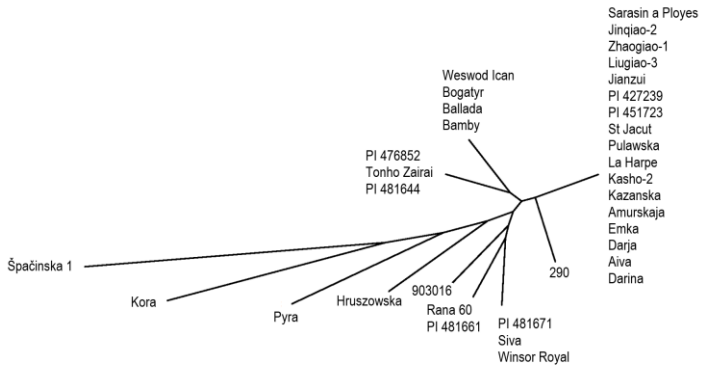

Figure 5 Dendrogram of obtained VBAP profiles for analysed buckwheat varieties/genotypes.

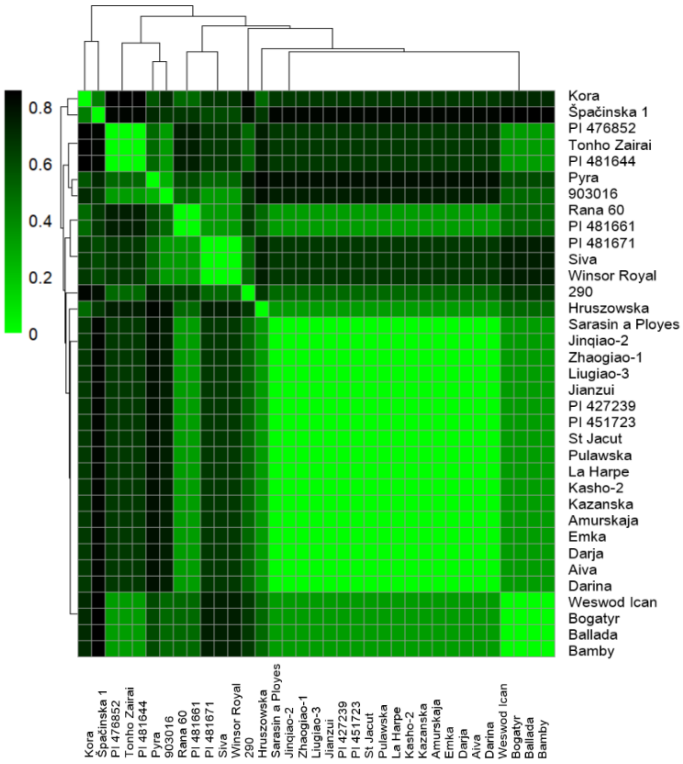

Figure 6 Heatmap of distance matrix of obtained VBAP profiles for analysed buckwheat varieties/genotypes.

Comparing all the used techniques, the ability of PBAP to detect polymorphism was comparable for BBAP and VBAP (table 1), but the ability of the primers to distinguish among the analysed varieties and genotypes was higher for BBAP. Discrimination power index was comparable for all of the used techniques.

Table 1 Characteristics of polymorphism generated by DNA marker techniques used in the study for buckwheat varieties/genotypes.

technique	H	PIC	E	MI	D	R
BBAP	0.43	0.34	2.86	0.004	0.9	2.97
PBAP	0.46	0.35	2.51	0.002	0.87	3.03
VBAP	0.43	0.34	2.46	0.004	0.91	2.06

H – heterozygosity index; PIC – polymorphism information content; E – effective multiplex ratio; MI – marker index; D – diversity index; R – discrimination power

For buckwheat species, different types of DNA based markers were employed previously. DNA polymorphism analysis was effective in buckwheat origins and phylogeny of the genus Fagopyrum research (**Tsuji and Ohnishi, 2000, 2001; Sharma and Jana 2002a, 2002b; Wang *et al.* 2004**). High-density maps linked with DNA markers were developed. **Aii *et al*.** (1998) linked RAPD markers to a self-pollinating gene; **Matsui *et al.*** (**2004**) prepared AFLP markers linked to a shattering gene; **Yasuo *et al.*** (**2004**) reported an genome-wide AFLP (amplified fragment length polymorphism) map for F2 progeny of hybrids between distylous self-infertile and the wild relative homostylous self-fertile *Fagopyrum esculentum* var. homotropicum; and integrated RAPD and STS linkage map of common buckwheat was reported by **Pan and Chen** (**2010**). Microsatellites markers were employed for population genetic analysis and the analysis of genetic diversity of common buckwheat (**Iwata *et al.* 2005, Konishi and Ohnishi 2007, Ma *et al.* 2009**), and EST (expressed sequence tags) markers were reported to identify candidate photoperiod-sensitivity genes for common buckwheat (**Hara *et al.* 2011**).

Here, two buckwheat species' genotypes and chosen varieties were analysed using the BBAP (Bet v 1 based amplified polymorphism), PBAP (profiling based amplification polymorphism), and VBAP (vicilin based amplification polymorphism) methodologies. High heterogeneity among plant species is revealed by *in silico* study of the amino-acid sequences that are available for the Bet v 1 allergen (**Breiteneder and Ebner, 2000**). This served as the basis for the use of these regions as DNA based markers for the analysis of plant DNA polymorphism. According to **Uehara et al.** (**2001**), the forward primer area of the BBAP approach has a comparatively high homology of amino acid sequences, including the proven epitope for IgE. Reverse primers match the amino acid variability and amplify a variable region of the year-10 gene of Bet v 1. Reverse primers match the amino acid variability at position 119 of the Bet v 1 protein (P15494) and amplify a variable region of the year-10 gene of Bet v 1 (**Breiteneder and Ebner, 2000**). This study examined several buckwheat accessions and

demonstrated the marker's ability to produce polymorphic fingerprints between them. In the past, intraspecific variability of *Malus domestica* Borkh. cultivars was investigated using degenerate primers that anneal a variable and conserved portion of PR-10 protein homologues genes (**Speváková *et al.* 2021**). The production of amplicons and their comparatively monomorphic profiles demonstrated the stability of the specified isoforms of Bet v 1 in the chosen apple cultivars.

Profilins, actin binding molecules belong to plant panaller-gens, what makes the PBAP universally in using (**Čerteková *et al*., 2023**). Plant profilin isoforms have minimal sequence divergence. Only flowering plants contain allergenic profilins. They represent a family of cross-reactive allergens in plant foods and monocot and dicot pollens because of their high degree of sequence conservation (**Radauer and Breiteneder, 2007**). Previously, PBAP was used to analyse the genotypes of groundnut and soybean in legumes (**Žiarovská *et al*., 2023; Kováčik *et al*., 2024**). Polymorphic profiles have been produced in both of these species. While all of the groundnut accessions could they be distinguished using a profiling-based marker approach, two soybean varieties produced fingerprint profiles that were identical. Higher plants encode a variety of profilin proteins, which are primarily classified into two groups according to how differently they express themselves in vegetative and reproductive tissues (**Kandasamy *et al*., 2002**). The NCBI (National Centre for Biotechnology Information) gene database currently shares information about more than 400 plant profilin proteins (**Jimenez-Lopéz *et al*., 2012**). According to the earlier research, even slight variations in the amino acid sequence can significantly alter the biochemical characteristics of profilin (**Ostrander *et al*., 1999**). Additionally, profiling genes with a polyphyletic evolutionary origin tend to have more diverse sequences (**Pandey and Chaudhary, 2020**).

Numerous plant species include vicilins, a class of allergenic seed storage proteins also known as 7S globulins. Because the Viciae group predominates in the legumes, they are frequently referred to as vicilins (**Astwood *et al*., 2002**). In buckwheta, stored endopeptidase was reported to start globulin mobilization in the cotyledons (**Belozersky *et al*., 1990**). It is generally known that allergens based on vicilin can cause cross-reactions with one another in legumes (**Arora *et al*., 2021**). Vicilins were employed as DNA markers in legumes analysis only previously (**Žiarovská *et al*., 2023; Kováčik *et al*., 2024**), this study is first where VBAP was applied to another group of plants.

CONCLUSION

Three allergen marker based techniques were used for the analysis of genetic diversity of selected *Fagopyrum ecsulentum* and *Fagopyrum tataricum* varieties and genotypes. In conclusion the result showed that all the allergen homology based marker techniques used here can be used to generate polymorphism in buckwheat germplasm, as they are highly informative according to the PIC value which was 0.34 or 0.35 respectively. None of the applied marker techniques is specie specific for buckwheat and reveals the polymorphism of allergen coding homologs relevant to the ancestral background of the genes per se, what can provide a practical information in breeding strategies of this species.

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