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BBAP Amplification Profiles of Golden Delicious and Granny Smith Apple Cultivars

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The BBAP analysis (Bet v 1 based amplified polymorphism) used in the study was aimed to detect natural variation among thirteen samples of Golden Delicious and Granny Smith apple cultivars. These cultivars were selected due to their global commercial importance and their documented association with high concentrations of the allergen Mal d 1. All of them were sourced from retail chains across eight European countries (Germany, Austria, Slovakia, Serbia, Croatia, Hungary, Italy, and Slovenia). Cross-reactivity between Bet v 1, a major allergen in birch pollen, and Mal d 1, its homolog in apples, often leads to allergic reactions in individuals sensitized to birch pollen. That is why determining the features of the genome of different cultivars of apples with the use of appropriate specific primers makes it possible to assess the possible allergenicity of products. Forward and reverse primers (R1, R2, R3, R4, Rd) were employed, and the resulting amplification products were analysed via electrophoresis on a 3% agarose gel. Most samples exhibited monomorphic patterns, suggesting similar isoform profiles, though some differences between cultivars were observed. A total of 73 fragments, ranging from 38 bp to 662 bp, were amplified. Profiles for primers FR3 and FR4 could not be successfully synthesized. For the primer pair FRd, fragment sizes ranged from 113 bp to 400 bp, with 26 fragments observed. Primer pair FR1 produced 20 fragments within a range of 181 bp to 662 bp, while primer pair FR2 resulted in 27 fragments ranging from 38 bp to 307 bp. All primer pairs exhibited the expected amplicon with a length of 388 bp. An average PIC value of 0.37 confirmed the high informativeness of the technique. The monomorphic profiles suggest that the environmental impact and varying storage conditions on the studied genes are minimal or negligible, as all samples exhibited similar profiles despite being sourced from diverse countries and retail stores. The identification of unique genetic profiles in certain regions or cultivars may provide insights into the allergenic potential of different apple cultivars, contributing to advancements in food safety, allergen management, and the development of hypoallergenic apple cultivars.

Keywords: BBAP, Bet v 1, variability, apple, Golden Delicious, Granny Smith

Introduction

The fruits of the *Malus × domestica* Borkh. (i.e., the apple tree) constitute a valuable source of nutrients, and thus a recommended component of a healthy, balanced diet. Consumption of apples has been linked with a decreased risk of various degenerative diseases, including cancer and cardiovascular disease

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(Kalinowska et al., 2014). It should be noted, however, that apples can also trigger allergic reactions, some of which can be severe. It is commonly observed that fruit allergies, including those to apples, are associated with the condition known as pollinosis. Based on the findings of several epidemiological studies, it is estimated that at least two million European citizens are affected by various fruit allergies. In North and Central Europe, the primary cause of apple allergy is cross-reactivity with birch pollen aeroallergens (Paris et al., 2017; Siekierzynska et al., 2021). The prevalence of allergic reactions to birch pollen in the European population has been reported to range from 8 to 16%. Among these individuals with allergies, a proportion of between 47 and 80% have been observed to exhibit cross-reactivity between birch pollen and apple allergens, with this proportion increasing in recent years. The underlying cause of this rising trend remains unclear, underscoring the growing need for the development of hypoallergenic fruits (Romer et al., 2020).

The differing expressions of Mal d 1, the primary allergen in Central and Northern Europe, may be responsible for the observed variations in allergenic potency among apple cultivars, indicating a significant genetic influence on Mal d 1 allergenicity. Additionally, the allergenic potential of apples may be influenced by the degree of ripeness, which correlates with the accumulation of Mal d 1 protein during the maturation process. Moreover, storage conditions and duration influence the accumulation of Mal d 1 and Mal d 2 allergens, as observed in the Golden Delicious and Granny Smith cultivars (Siekierzynska et al., 2021). This study was aimed to analyse the results of BBAP reactions to identify and characterise the polymorphism of the Mal d 1 gene in two commercially important apple cultivars, Golden Delicious and Granny Smith. These cultivars are widely consumed worldwide, making them relevant for allergenicity studies aimed at improving food safety and consumer health.

Material and methodology

Biological Material

In the present study, the biological material consisted of fruits from two apple cultivars, namely: Golden Delicious and Granny Smith (*Malus domestica* Borkh.). A total of 13 samples (Table 1) were sourced from retail chains across several European countries, including Germany, Austria, Slovakia, Serbia, Croatia, Hungary, Italy, and Slovenia. It should be noted that the samples were not grown, harvested, or stored in the same location or at the same time, and therefore were likely subjected to different growth and storage conditions. In order to maintain their properties, the samples were stored frozen until the analysis.

DNA extraction

The total genomic DNA was extracted using the Eligene Plant Genomic DNA Purification Kit (Elisabeth Pharmacon), following the methodology described in the manufacturer's instructions. The extracted DNA was analysed utilising the Nanophotometer P360 (Implen) in order to evaluate its concentration and purity. The quality of the extracted DNA was additionally assessed through polymerase chain reaction (PCR) using internal transcribed spacer (ITS) primers, as designed by White et al. (1990). The PCR was performed on an Agilent SureCycler 8800.

BBAP analysis

To ascertain the presence of homologs of the Bet v 1 gene, a polymerase chain reaction was conducted using EliZyme HS Robust MasterMix (Elisabeth Pharmacon). The reaction mixture (10 µl) comprised 5 µl of MasterMix, 0.4 µl of the forward primer (F), 0.4 µl of the reverse primer, 2.2 µl of water, and 2 µl of the extracted DNA. The samples were diluted in a 1:1 ratio. Negative controls without DNA template were included to ensure the specificity of the reaction. The following primers were employed (Žiarovská and Urbanová, 2022):

- Forward Primer:
    - F: 5' CCT GGA ACC ATC AAG AAG 3'
- Reverse Primers:
    - Rd: 5' TTG GTG TGG TAS TKG CTG 3'
    - R1: 5' TTG GTG TGG TAG TGG CTG 3'
    - R2: 5' TTG GTG TGG TAG TTG CTG 3'
    - R3: 5' TTG GTG TGG TAC TGG CTG 3'
    - R4: 5' TTG GTG TGG TAC TTG CTG 3'

The polymerase chain reaction (PCR) was conducted on the Agilent SureCycler 8800 by the following conditions: an initial denaturation at 95 °C for 5 minutes, followed by 40 cycles of denaturation at 95 °C for 45 seconds, annealing at 54 °C for 45 seconds, and elongation at 72 °C for 35 seconds. The reaction was then terminated with a final elongation step at 72 °C for 10 minutes.

Table 1 List of samples with respective codes and country of origin

Code	Cultivar	Country of origin
MD1z	Golden Delicious	Germany
MD2z	Granny Smith	
MD3z	Golden Delicious	Austria
MD4z	Granny Smith	
MD5z	Golden Delicious	Slovakia
MD6z	Granny Smith	
MD7z	Golden Delicious	Serbia
MD8z	Granny Smith	
MD9z	Golden Delicious	Croatia
MD10z	Granny Smith	
MD11z	Golden Delicious	Hungary
MD13z	Golden Delicious	Italy
MD15z	Golden Delicious	Slovenia

The PCR products were visualised by electrophoresis on a 3% agarose gel stained with GelRed™. Several ladders were used, for the primer pair F+R1 the 100 bp DNA Ladder ready to use (Bioron life science) and for F+Rd and F+R2 PRC Biosystems PCRBIO Ladder III (PCR biosystems).

Statistical analysis

BBAP fingerprints were analyzed by GelAnalyzer 23.1.1 (www.gelanalyzer.com) and the matrixes were created based on the presence or absence of specific loci. Graphs were prepared using Microsoft Office Excel 2021 (WINDOWS 11).

Results and discussion

In order to gain a deeper insight into the genetic basis of allergen production among apple cultivars, molecular markers, and DNA fingerprinting techniques have been employed. These techniques have been developed to identify and analyse polymorphism in a cultivar of plants, including the assessment of genetic diversity. These markers are valuable tools for the creation of genotype-specific DNA profiles, the tagging of genes for the selection of suitable genotypes, the enhancement of breeding efficiency, and the distinction of similar plant cultivars at the DNA level. Genetic markers facilitate the identification of differences at the DNA level, thereby enabling the effective distinction between similar plant cultivars. A number of different molecular markers are available for the assessment of genetic diversity in plant species (Bilčíková et al., 2021; Spevákova et al., 2021). Molecular or DNA markers are becoming increasingly prevalent in basic genomic studies and applied plant breeding. The choice of molecular marker is dependent on a number of factors, including the plant species under investigation, the objectives of the research project, and the availability of necessary resources. Codominant markers, distinguish between genotypes, possess a multiallelic nature, are abundant in the genome, and are specific to the genus or species of interest are particularly preferred (Amiteye, 2021; Oliveira et al., 2022).

BBAP (Bet v 1 homologs-based amplified profile) is a DNA marker technique that employs the homology of *ypr10* genes, which are genes encoding the primary allergen of birch pollen. The extensive distribution of these homologous genes across plant species provides a foundation for the functionality of DNA markers based on their sequences. This technique allows for the detection of *ypr10* genes and a number of their variants within plant genetic material. The utilisation of specific degenerate primers enables the screening and identification of the presence of *ypr10* genes, which are responsible for the production of PR-10 proteins and may therefore be potential allergens. Furthermore, BBAP is employed to investigate the genetic relationships between different plant species with homologous Bet v 1 genes (Žiarovská and Urbanová, 2022).

In our BBAP analysis, the overall size range for both the entire sample set and the Golden Delicious samples was observed to be between 38 and 662 base pairs (bp), while for Granny Smith, it ranged between 45 and 400 bp. The size ranges of the fragments indicate a degree of similarity in the sequences between individual samples of Granny Smith and Golden Delicious. No synthesised fragments were identified in the Golden Delicious sample MDz3.

Although the concentration of Mal d 1 allergen in apples is known to vary, the electrophoresis results indicated that all samples were monomorphic. This monomorphism indicates that the isoform profiles of the *ypr10* gene are consistent across all samples. The synthesis of the profiles for primers FR3 and FR4 was unsuccessful. The PIC value for the F+Rd primer was 0.368, while for primers F+R1 and F+R2, the value was slightly higher, at 0.374. These values indicate that the individual markers within the analysed sample set are highly informative.

The F+Rd primer pair was employed to assess the amplicon sizes of the samples (Figure 1). The amplicons were found to be of three defined lengths: approximately 396 bp, 212 bp, 152 bp, and a fragment of approximately 95 bp. The 396 bp fragment was consistently amplified in all Golden Delicious samples, but was absent in Granny Smith samples MDz2, MDz6, and MDz10. The 212 bp fragment was successfully amplified across all samples. Furthermore, a 152 bp fragment was observed in samples MDz2 and MDz3, and a 113 bp fragment was present in sample MDz13.

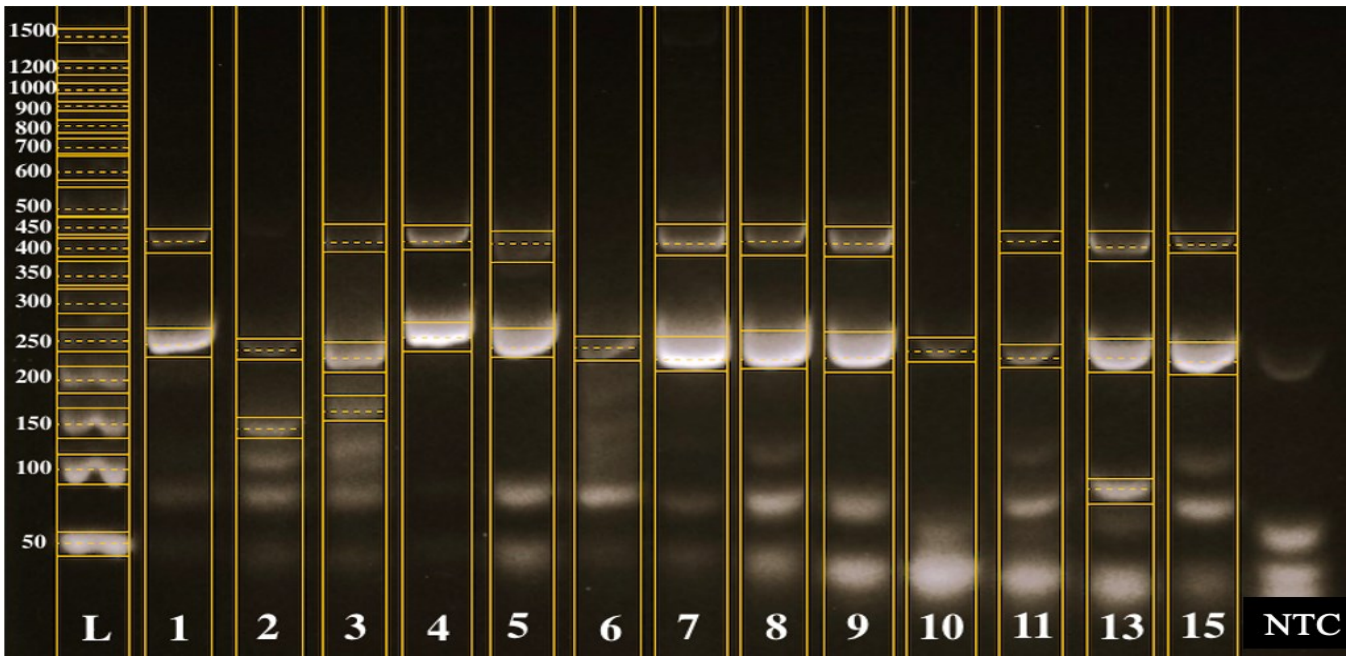

Figure 1 BBAP, primers F+Rd (from left to right: ladder, samples MDz1-MDz15, NTC – no template control) Ladder PRC Biosystems PCRBIO Ladder III (PCR biosystems) was used.

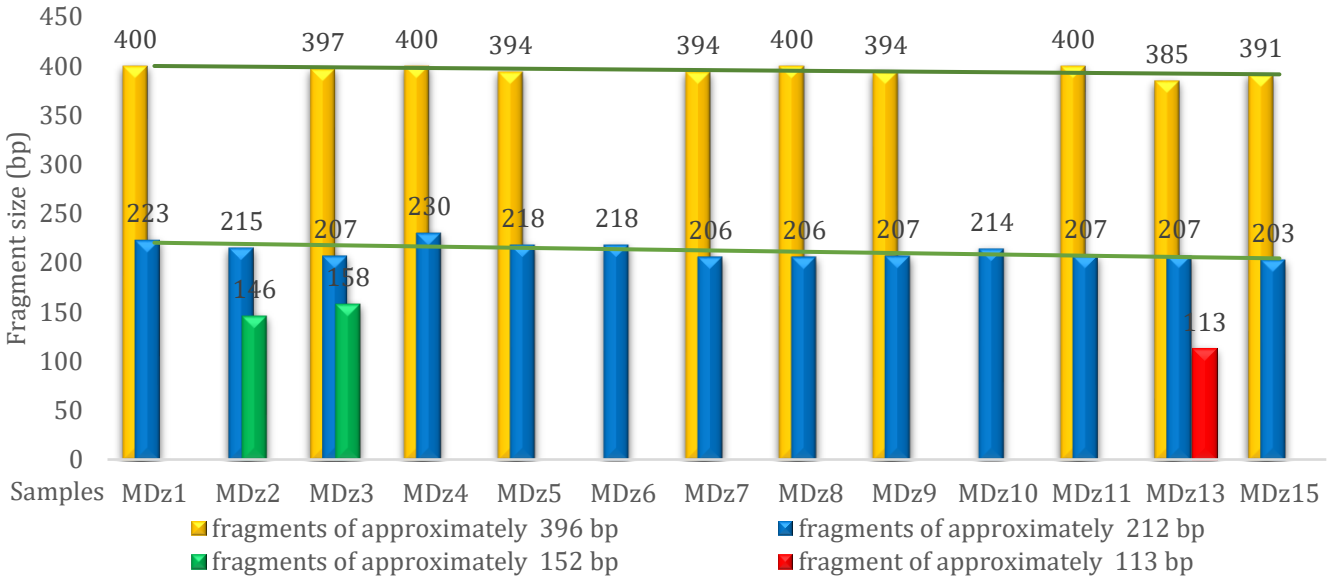

Figure 2 Distribution of Fragment Sizes Amplified in BBAP Analysis Using F+Rd Primers in Samples MDz1-MDz15. Values ale absolute.

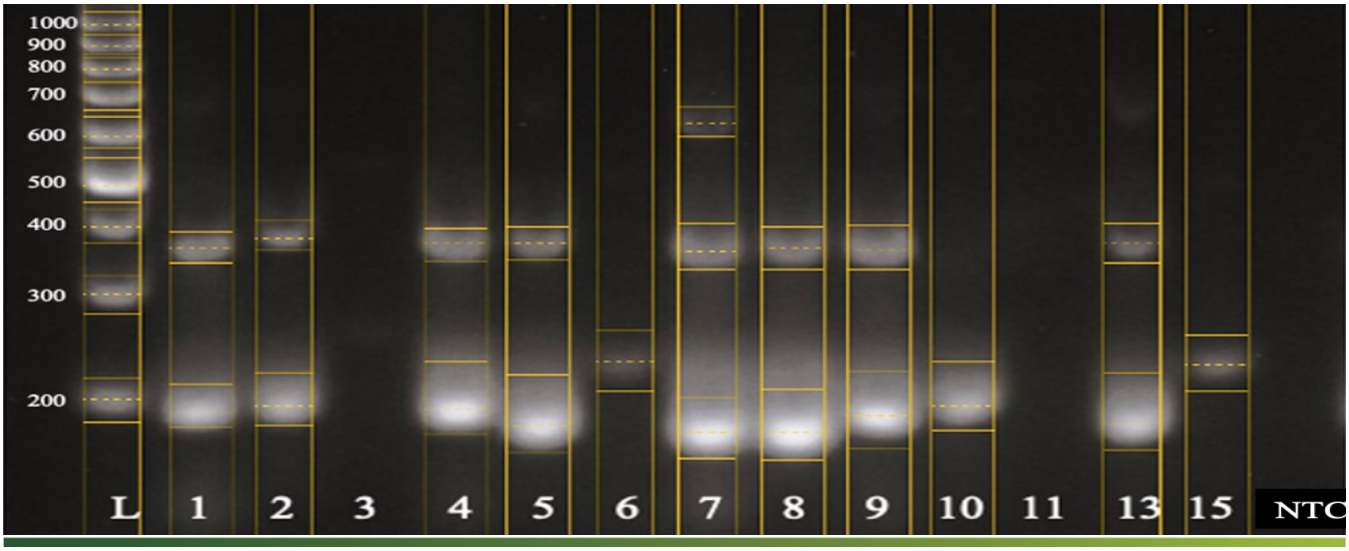

Figure 3 BBAP, primer pair F+R1 (from left to right: ladder, samples MDz1-MDz15, NTC -no template control). Ladder 100 bp DNA Ladder ready to use (Bioron life science) was used.

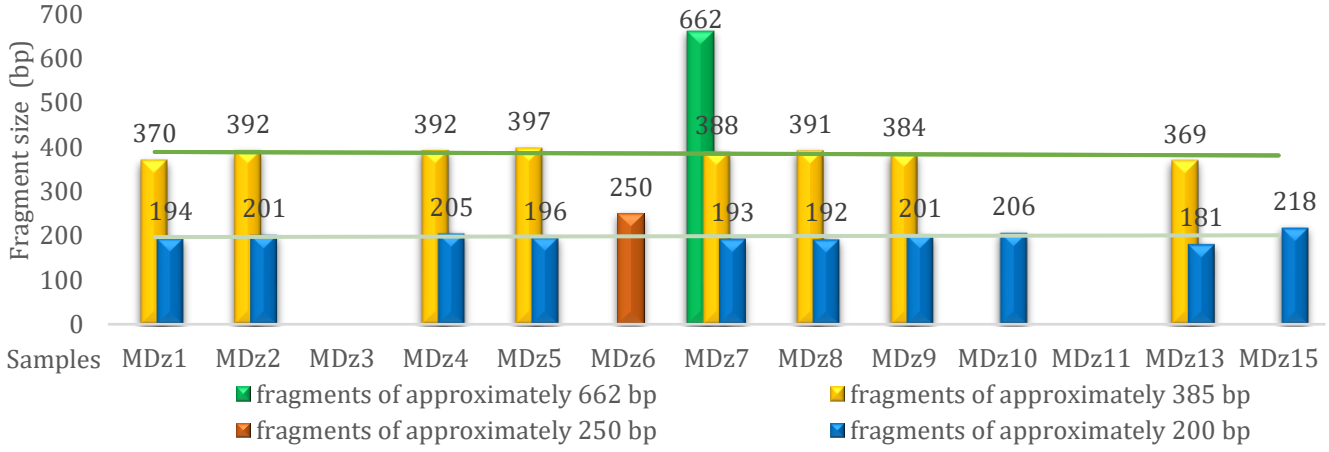

Figure 4 Distribution of Fragment Sizes Amplified in BBAP Analysis Using F+R1 Primers in Samples MDz1-MDz15. Values ale absolute.

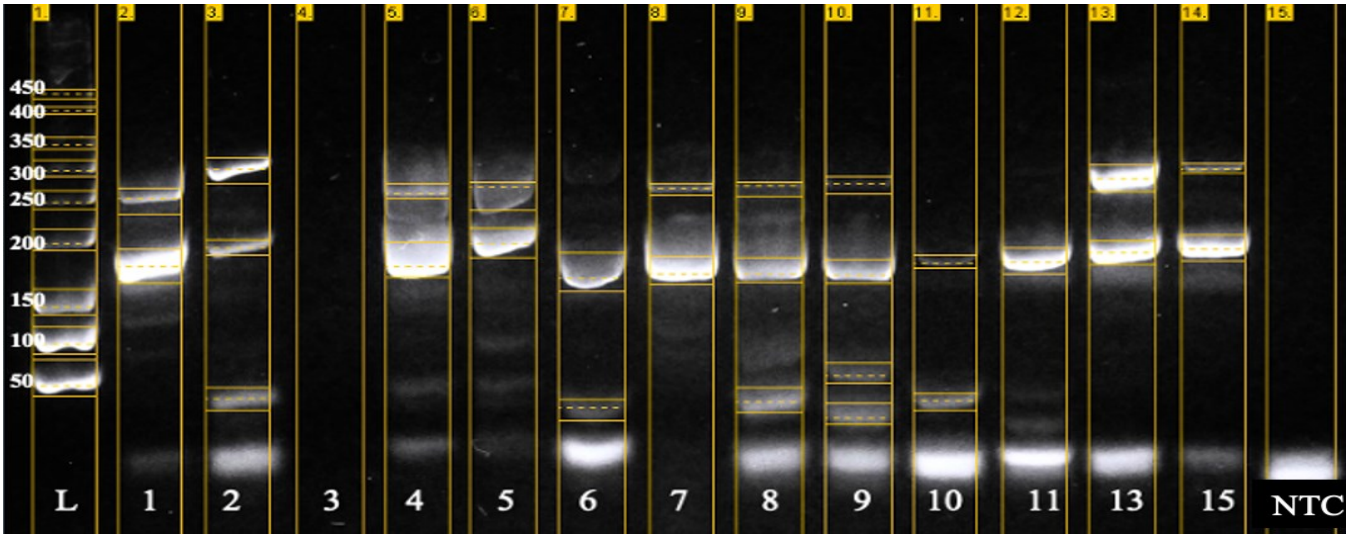

Figure 5 BBAP, primers F+R2 (from left to right: ladder, samples MDz1-MDz15, NTC - no templatecontrol). Ladder PRC Biosystems PCRBIO Ladder III (PCR biosystems) was used, fragments bigger than 450 are not clearly visible.

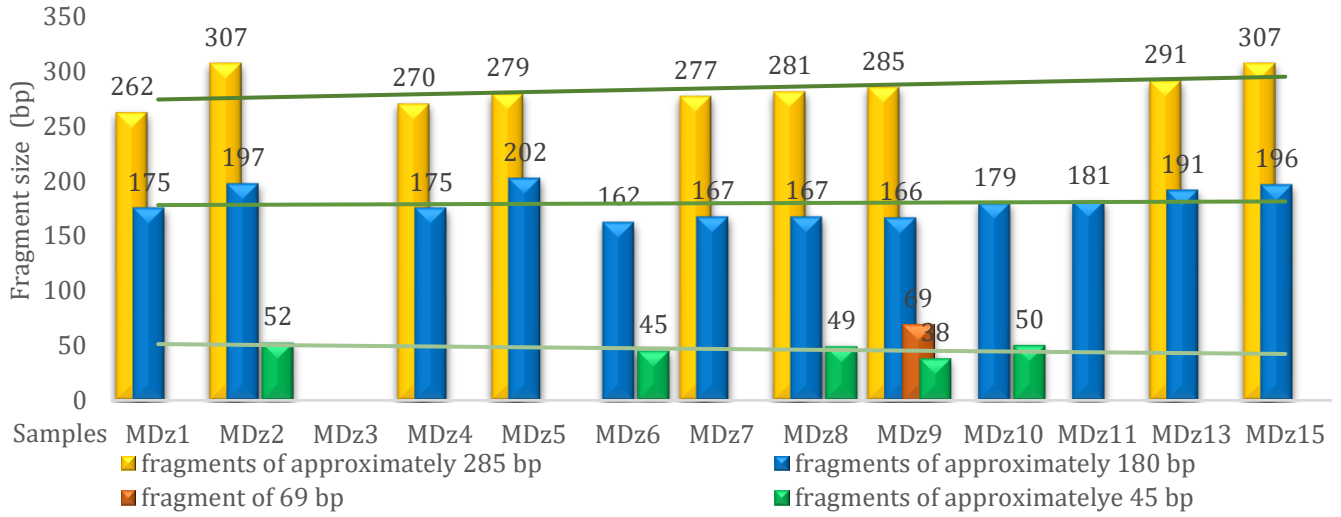

Figure 6 Distribution of Fragment Sizes Amplified in BBAP Analysis Using F+R2 Primers in Samples MDz1-MDz15. Values ale absolute.

The analysis of fragment sizes across both Granny Smith and Golden Delicious cultivars revealed ranges of 146 bp to 400 bp for Granny Smith and 113 bp to 400 bp for Golden Delicious (Figure 2). The entire sample set exhibited fragment sizes between 113 bp and 398 bp.

A total of 26 fragments were amplified. A total of 18 fragments were amplified in Golden Delicious samples and 8 in Granny Smith samples, including the 396 bp, 212 bp, 152 bp (MDz2, MDz3), and 113 bp (MDz13) fragments.

As illustrated in Figure 3, the F+R1 primer pair was used to amplify a total of 20 fragments. The synthesis of fragments for the cultivar Golden Delicious yielded a range of 181-662 bp, encompassing the full range observed in all samples. In the case of the cultivar Granny Smith, the fragments in question exhibited a range of 192 to 392 base pairs. No amplification of fragments was observed in samples MDz3 and MDz11.

The most frequently synthesised fragments were approximately 200 bp in size (ranging from 181-218 bp), amplified in nine samples (MDz1, MDz2, MDz4, MDz5, MDz7-10) (Figure 4). The second most frequently synthesised fragment was approximately 385 bp in size (ranging from 369-397 bp), amplified in eight samples (MDz1, MDz2, MDz4, MDz5, MDz7-9, MDz13, and MDz15). The stability observed in fragment size between samples suggests that the isoform profiles captured by the BBAP technique are consistent across the sample set. Unique fragments were observed in MDz6 (~250 bp) and MDz7 (the largest fragment at 662 bp).

The samples were observed to exhibit monomorphism, except MDZ3 and MDZ11, where amplification was unsuccessful. Deviations from other samples were noted in MDZ6 and MDZ15, where fragments of approximately 250 bp and 662 bp, respectively, were identified.

The use of primers F+R2 resulted in the amplification of a total of 27 fragments, with 15 observed in Golden Delicious samples and 12 in Granny Smith samples (Figure 5).

Most samples exhibited the presence of fragments with an approximate length of 285 bp, with fragment sizes ranging from 262 bp to 307 bp (Figure 6). Fragments of approximately 180 bp were identified in all samples. A fragment of approximately 69 bp was identified exclusively in MDz9. Fragments of approximately 45 bp were predominantly amplified in Granny Smith samples (MDz2, MDz6, MDz8, and MDz10) and one Golden Delicious sample (MDz9).

The functionality of the BBAP technique has been confirmed for a range of fruit species, demonstrating its potential as a universal marker system applicable to all plant species for screening Bet v 1 homologs and the similarity of their fingerprints (Urbanová and Žiarovská, 2021). In the study by Žiarovská and Urbanová (2022), all results included amplicons of 388 base pairs in length. In addition, primers F+R1 revealed the presence of amplicons measuring 217 base pairs (bp). The analysis of the selected apple cultivars revealed similar fragment lengths. Speváková et al. (2021) employed a degenerate primer pair to amplify a total of three amplicons, with lengths of 195,

400, and 705 base pairs, respectively. The sample profiles (Spencer, Ecollete, Melrose, Jonalord, Angold, Sonet, Ligol, Kinova, Gala, and Pink Lady) exhibited notable similarity, forming a single polymorphic and two monomorphic profiles. A comparison of the results presented here with those of Žiarovská and Urbanová (2022) and Speváková et al. (2021) reveals a high degree of consistency in the generation of fragments, thereby reinforcing the reliability of the BBAP analysis.

In a study conducted by Moravčíková and Žiarovská (2023), the BBAP technique was employed to amplify DNA fragments within *Citrus sinensis* cultivars, resulting in the revelation of notable differences concerning the number and size of the amplified fragments. Primers R2, R3, and R4 were observed to detect Bet v 1 homologs at approximately 388 base pairs. Bet v 1 homologs have been identified in a wide range of plant species, including *Citrus sinensis*, which highlights their role in allergic reactions. Furthermore, the BBAP technique was employed for the amplification of profiles in *Avena sativa* L. by Farkasová et al. (2023). The Bet v 1-based amplified profile (BBAP) method revealed polymorphic profiles with amplicons ranging from 265 to 1929 base pairs in seven oat cultivars. The study revealed the presence of unique amplicons in each cultivar, with no evidence of shared size among all cultivars.

Plants utilise PR proteins to defend against a range of stresses, with the expression of these proteins being triggered by a cultivar of factors, including abiotic and biotic stresses. These factors encompass pathogen invasion, cold, salinity, drought, oxidative stress, UV radiation, and physical damage. Although PR-10 proteins lack a distinct, singular function, they play a general role in plant development and defence mechanisms. A common feature of PR-10 proteins is that they are encoded by multiple genes, referred to as *ypr10* genes. Bet v 1-like proteins are small (consisting of 154–163 amino acid residues), possess a slightly acidic character, are primarily cytoplasmic in location, and have a molecular mass of approximately 17 kDa. They are typically susceptible to digestion in the stomach and heat-induced denaturation, which diminishes their capacity to elicit IgE responses (Pühringer et al., 2000; Sancho et al., 2011; Aglas et al., 2020; Breiteneder and Heimo, 2023).

The prevalence of sensitisation to Mal d 1, the major allergen found in apples, often mirrors that observed with Bet v 1 from birch (*Betula verrucosa* Roth) pollen, with rates ranging from 53 to 95% among individuals with birch pollen allergy. This similarity can be attributed to the IgE-mediated cross-reaction with Bet v 1, the major birch pollen allergen, with which Mal d 1 shares a high degree of homology (Paris et al., 2017; Aglas et al., 2020). A comparison of the amino acid sequences of Mal d 1 and Bet v 1, the major birch pollen allergen, revealed a 64.5% identity and a 55.6% identity at the nucleic acid level (Vanekkrebitz et al., 1995).

It has been demonstrated that high concentrations of Mal d 1 are commonly found in cultivars of apples such as Granny Smith and Golden Delicious (Fritsch et al., 1998). In the context of food allergy management, the removal of allergenic food from the diet is of the utmost importance and may, over time, result in a reduction in sensitisation. Nevertheless, in the case of the birch-apple syndrome, the continuous exposure to Bet v 1 throughout the birch pollen season renders the likelihood of a reduction in sensitisation to the cross-reactive Mal d 1 allergen remote (Incorvaia et al., 2017).

It should be noted that the concentration of Mal d 1 in apples can vary depending on the specific apple culti and is susceptible to fluctuations due to various environmental and storage conditions. The concentration of Mal d 1 in apples is typically low (approximately 1-30 µg per gram) at the time of harvest. However, storage conditions can significantly influence the accumulation of Mal d 1 and result in levels exceeding 100 µg per gram. Analyses have demonstrated that Mal d 1 and Mal d 2 are present throughout both the apple pulp and peel, whereas Mal d 3 is confined to the peel. The peel of apples has been observed to contain higher concentrations of this protein than the pulp. Given that Mal d 1 expression is increased in response to biotic stress, this protein probably plays a role in the apple's defence against pathogens (Marzban et al., 2005; Matthes and Schmitz-Eiberger, 2009; Schmitz-Eiberger and Matthes, 2011; Ahammer et al., 2017).

Conclusions

The objective of the BBAP marker analysis was to assess the genetic variability and polymorphism within the coding regions of the apple cultivars under study. The BBAP system was employed as a universal marker system for the screening of Bet v 1 homologs and the analysis of the similarity of their patterns. The electrophoresis results indicate monomorphism among the samples, with the majority displaying comparable isoform profiles of the *ypr10* gene. A total

of 73 fragments were amplified, with sizes ranging from 38 bp to 662 bp. The average PIC value (0.37) also demonstrates the high informativeness of the marker technique employed.

Conflict of interest

The authors declare no conflict of interest.

Ethical statement

This article doesn't contain any studies that would require an ethical statement.

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