Comparison of the of BBAP and CDDP in *Fragaria* sp. Polymorphism Analysis

Silvia Farkasová[1], Lucia Urbanová[1], Jana Žiarovská[2], Matúš Kyseľ[3], Alžbeta Jauschová[2], Lenka Kučerová[2], Miroslava Kačániová[4]

[1]AgroBioTech Research Centre, Slovak University of Agriculture in Nitra, Tr. A. Hlinku 2, 949 76 Nitra, Slovak Republic
[2]Institute of Plant and Environmental Sciences, Faculty of Agrobiology and Food Resources, University of Agriculture in Nitra, Tr. A. Hlinku 2, 949 76 Nitra, Slovak Republic
[3]Department of Dendrobiology, Institute of Forest Ecology, Slovak Academy of Sciences - Zvolen, Vieska nad Žitavou 178, 951 52 Slepčany, Slovak Republic
[4]Institute of Horticulture, Faculty of Horticulture and Landscape Engineering, Slovak University of Agriculture in Nitra, Tr. A. Hlinku 2, 949 76, Nitra, Slovak Republic

Abstract
Strawberries are popular worldwide for their nutritional benefits. Their germplasm is widely analysed by different molecular and DNA based markers. Here, two different marker techniques were used to analyze intra- and interspecies variability of 38 genotypes of two strawberry species (*Fragaria* x *ananassa* and *Fragaria vesca* L.). Bet v 1 based amplified polymorphism (BBAP) and conserved DNA-derived polymorphism (CDDP) utilized to obtain specific fingerprints. BBAP technique generated lower polymorphism comparing to CDDP (BBAP – 99.01%, CDDP – 99.65%), but both of the techniques separated all of the analysed strawberry genotypes. The groups of genotypes in constructed dendrograms were not clustered according the specie specificity.

Keywords: strawberries, BBAP, CDDP, polymorphism.

1. Introduction

Plants have a dynamic, innate immune system that responds to and/or protects against different stress factors based on the development of multifunctional components with additional, minor, lateral or marginal functionality as the defence-related proteins [1-3]. One of the group of defence-related proteins, PR-10 (pathogenic related) were characterized in fruits as allergens [4]. The abundant Bet v 1 (birch main pollen allergen) homologs of the PR10-proteins group have approximately 160 aminoacids with a molecular weight about 17 kDa and a pH of 4.5–6 [5]. Strawberry homolog of this allergenic group, Fra a 1 proteins, belong to the Bet v 1-related PR-10 proteins and are the most closely sequence-related ones to the proteins Pru av 1 of cherries (79%) [6], while the similarity to Bet v 1 is about 70% depending on isotype and isoform [7]. Yang et al. [3] recognized up to 13 isotypes of the *Fra a 1* gene, of which Fra a 1.03, 1.04, 1.05, Fra v 1.01 and 1.08 are proteins with confirmed allergenic potential. Structure of Fra a 1 protein in the presence and absence of flavonoids allowed crystallization and acted as a monomer in a solution [8]. Plant allergens share a high degree of sequence homology for proteins, as well as for genomic sequences [9,10], what allow to predict DNA markers for them by *in silico* approach.
Previously, BBAP 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 epitopes in plants

* Corresponding author: Jana Žiarovská, +421376414244, jana.ziarovska@uniag.sk

[11,12]. The technique amplify polymorphic sequences of Bet v 1 homologs and the primers anneal the epitope by genenerate reverse primer [13].
A CDDP technique anneals conservative parts of typical functional domains of genes across plant species [14]. In this study, WRKY genes involved in developmental and physiological processes where chosen by their primers. WRKY genes belong to a gene family found in a large number of higher plants [15] and act in an expression processes of response to biotic and abiotic stress, aging or dormancy [1,16-19]. Numerous members of the WRKY gene family have been identified in varieties of plant species such as from corn, soybean, rice, apple, poplar, cassava, grapevine, bean, potato, false brome, tomato, *Arabidopsis thaliana*, mulberry or carrot as Khoso et al. [20] summarised.
To our knowledge, any of this two DNA based techniques was not used yet to characterize the polymorphism variability for *Fragaria* spp.

The aim of the study was to analyse the fingerprints of *Fragaria* spp genotypes by Bet v 1 based amplified polymorphism and by conserved DNA-derived polymorphism, compare them as well as verify their applicability for this specie.

2. Materials and methods

Biological material selected for analyses consists of 38 genotypes of two strawberry species (*Fragaria* X *ananassa* and *Fragaria vesca* L.) collected from in situ in different regions (Table 1). Mature leaves were used for bulk DNA extraction of genomic DNA using the GeneJET^{TM} Plant Genomic DNA Purification Mini Kit. Quality and quantity of extracted DNA was verified by spectrophotometric determination using NanoPhotometer^{TM} (IMPLEN). Functionality of DNA was checked by PCR amplification of Internal transcribed spacer [21].
For both, BBAP and CDDP reactions a mixture of MasterMix EliZyme HS Robust MIX was used following the time and temperature protocol: 95 °C for 5 minutes, followed by 40 cycles of denaturation at 95 °C for 45 s, annealing of primers at 54 °C for 45 s, and polymerization at 72 °C for 35 s with final polymerization at 72 °C for 10 minutes. Obtained amplicons were separated in 10% polyacrylamide gels. Four sets of molecular markers [12] were applied in the case of BBAP and five sets for CDDP technique [14].
Obtained fingerprints were analysed using the software GelAnalyzer 1.0 for image analyses, and transformed into a binary matrix. UPGMA based dendrograms were constructed based on Jaccard´s coefficient, and set of other statistical tools ANOVA, AMOVA, correlation, and polymorphism calculations in MS Excel.

3. Results and discussion

A set of 38 strawberry genotypes was analysed for their polymorphism by BBAP and CDDP techniques. Using one-way ANOVA with the assumption of generated binary matrices showed that the BBAP technique generated lower polymorphism comparing to CDDP (BBAP – 99.01%, CDDP – 99.65%). For CDDP, individual polymorphism of the profiles of primer pairs was as follows: F/R1 - polymorphism 100%, F/R2a - polymorphism 99.43%, F/R3a – polymorphism 99.61%, and F/R3b – polymorphism 100% (Figure 1). Polymorphism at the level of genotypes of the BBAP marker technique covered by the F/R_{deg} primer pair was 98.51% and for individual primer pairs as follows: F/R1 – polymorphism 98.51%, F/R3 and F/R4 – both polymorphism 100%, and at the F/R2 – polymorphism 96.55% (Figure 2). In relation to the botanical species of strawberry, the variability is related preferably to *F.* x *ananassa* and only in 2 cases to *F. vesca*. The consistency of the CCDP markers was confirmed by the compact output of all primer pairs in contrast to the BBAP markers. The binary profiles of the primer pairs F/R1, F/R2, F/R3, and F/R4 of the BBAP marker technique are variable among ANOVA outputs and the correlations was only in the case of F/R2. In this case, the binary profile of F/R_{deg} differed the same as was in the case of the ANOVA analysis. Correlation by the CDDP technique, in contrast to BBAP, turned out to be significantly more compact across primer pairs. The F/R2b primer pair had a slightly different binary profile by ANOVA while the F/R2a profile became the different one in the correlation analysis. This is the only case of CDDP markers when the slight negative correlation between the monitored genotypes exceeded 25%. In relative values, the binary profiles of the F/R3b primer pair genotypes behaved strongly-positive correlated.

Constructed dendrograms shown in both of the used techniques a very good reliability with the cophenetic correlation coefficients that differs only by tenths of a percent (CDDP – 97.30%, BBAP – 97.00%). A more detailed examination of the binary matrices of individual primer pairs recorded more significant differences (BBAP: F/R_{deg} – 98.31%, F/R1 – 89.65, F/R2 – 97.44, F/R3 – 86.70, F/R4 – 91.15; CDDP: F/R1 – 81.10, F/R2a – 90.80, F/R2b – 85.73, F/R3a – 85.23, F/R3b – 99.13). Both techniques identified 4 consistent clusters that form the skeleton of both cumulative dendrograms (Figures 3a and 3b). While the CDDP primers recognized 10 genotypic pairs, 10 genotypic clusters, and 1 non-specific grouping of genotypes; only 7 genotypic pairs, 5 genotypic clusters, and up to 3 non-specific genotype groupings were captured on the BBAP dendrogram. Both plots directly match only in 2 pairs of binary profiles (FA1-FA2 and FA12-FA13). The difference in the location of other genotypes lies in the interaction patterns with genotypes captured by only one marker technique (BBAP – FV4 and FV10, CDDP – FV1, FV7, FV11, FV14, FV18, FV 20 and FV22).

An AMOVA analysis of all 38 strawberry genotypes was performed to compare distribution of genetic variability between marker techniques. A variability captured with ANOVA was expected and supported with polymorphism and correlation should be one-sided in favor to interspecies than intraspecies differences. The BBAP technique does not recognize *F.* X *ananassa* and *F. vesca* as 2 separated OTU with 2 of 5 primer pairs (F/R_{deg} and F/R4). All the other primer pairs monitored differences among analysed species below 10% except F/R2 where the molecular variance reached 17% among analysed species. The same results provided AMOVA for CDDP technique (primer pairs F/R1 and F/R3a without species recognition, 2 primer pairs below 10% and FR2b 14% of the molecular variance among analysed species. Only 2 CDDP primer pairs recognized these species below 5% of molecular variance among.

Table 1. List of analysed genotypes of strawberries

Fragaria vesca		*Fragaria* X *ananassa*	
FV1	Nitra-Zobor	FA1	Victoria
FV2	Skalka	FA2	White dream
FV3	Donovaly	FA3	Selva
FV4	Demänovská Valley	FA4	Senga Sengana
FV5	Liptovský Ján	FA5	Honeoye
FV6	Plitvice	FA6	Korona
FV7	High Tatras	FA7	Elkat
FV8	Nová Baňa – Kostivrch	FA8	Wendy
FV9	Harmanec Cave – Veľká Fatra	FA9	Ostara
FV10	Lednice	FA10	Polka
FV11	Bojnice	FA11	Florence
FV12	Duchonka	FA12	Anabel
FV13	Nitra-Krškany	FA13	Symphony
FV14	Staré hory	FA14	Elsanta
FV15	Špania dolina	FA15	Sonata
FV16	Turčianske Teplice	FA16	TAgo
FV17	Dubnica nad Váhom		
FV18	Svatý Štepán		
FV19	Hranice		
FV20	Olomouc		
FV21	Pravenec		
FV22	Horné Lefantovce		
FV23	Červený Kláštor		

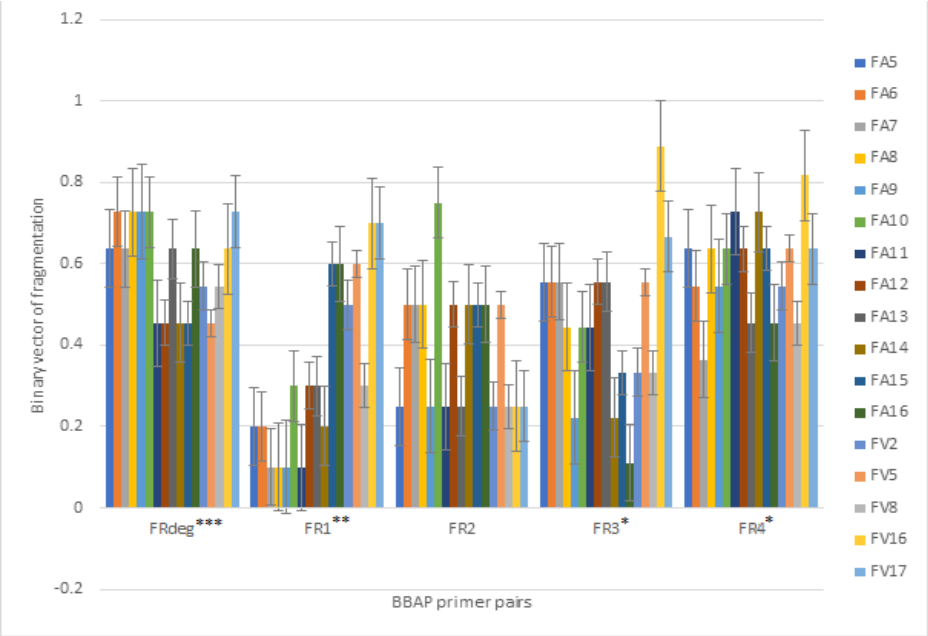

Figure 1. Variability of binary fragmentation vectors of BBAP profiles analysed by ANOVA. DNA fragmentation rate is expressed as mean ± SE. Legend to the graph on the left. * Variability at the separation level (P<0.001). ** Variability at the population level (P<0.01). *** Variability at the genotype level of both FV (P<0.01) and FA (P<0.001) groups.

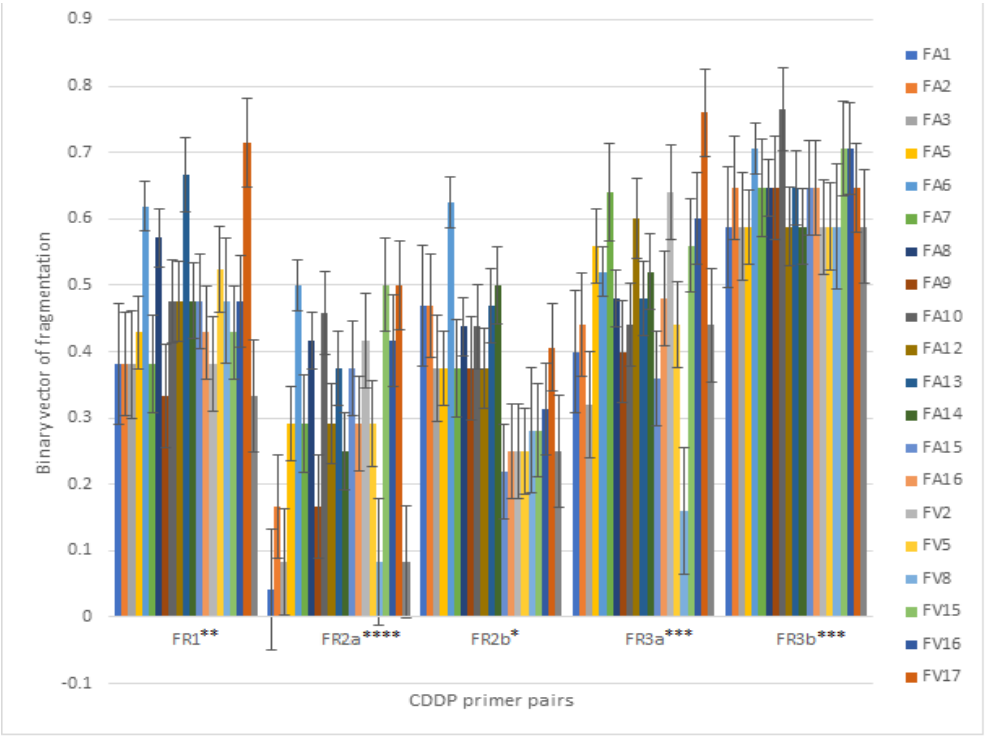

Figure 2. Variability of binary fragmentation vectors of CDDP profiles analysed by ANOVA. Legend to the graph on the right (b). * Variability at the separation level (P<0.001). ** Variability at the level of FA genotypes (P<0.01). *** Variability at the level of FA genotypes (P<0.001). **** Variability at the genotype level of both FV (P<0.01) and FA (P<0.001) groups.

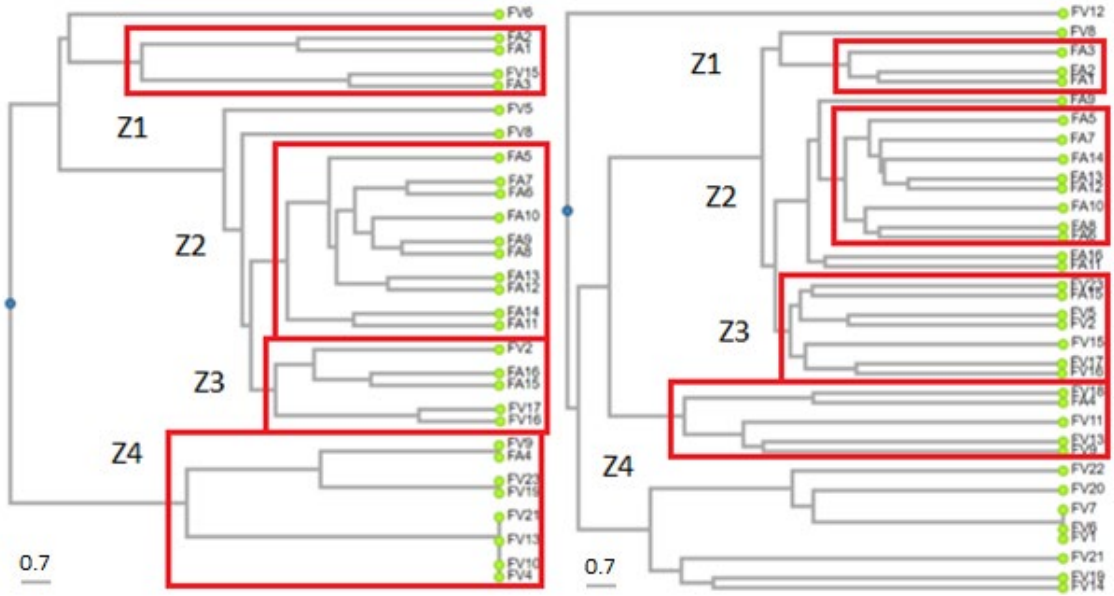

Figure 3. Dendrograms of CDDP(a) and BBAP (b) profiles of analysed strawberry genotypes. Dendrograms were constructed by cumulating binary matrices of individual primer pairs. The DendroUPGMA online tool was used to create dendrograms plots with the Jaccard similarity index and UPGMA clustering method. BBAP is posed on the left, CDDP on the right side. Consistent clusters recognized by both techniques: Z1 – FA1, FA2 and FA3; Z2 – FA5, FA6, FA7, FA8, FA10, FA12, FA13, and FA14; Z3 – FA4, FV9 and FV13; and Z4 – FA15, FV2, FV16 and FV17.

The assessment of genetic diversity and evaluation of the genetic relationships are a valuable source of information for conservation strategies and breeding programs. The strategies have a particular role in characterization of individual cultivars, in revealing the duplications of genetic material in germplasm collections and as a useful guide for selection of parents for breeding [22]. Various types of molecular markers have been applied for the assessment of fruit species but no single technique can be universally ideal. The choice of a technique is dependent upon the objective, skills and financial or technical possibilities and constraints [23,24]. Report of a new DNA-based marker technique (BBAP) aimed to allergen was announced to be a potential universal marker system that is usable throughout the fruit species to screen Bet v 1 homologs fingerprints similarity [12]. Bet v 1 homologs have significant IgE-induced activity after mutual cross-reaction with a multisymptomatic manifestation, which may not be recognized visually. The variability of allergen affection and its activation are largely influenced by the cause of the allergic reaction, the plant species responsible for transport and accumulation [25]. It is a reaction to infectious sites with propagation of bacterial, viral and fungal colonies in which Bet v 1-related proteins accumulate followingly. In this study no concrete isoforms of Fra a 1 and Fra v 1 allergens were analysed, but all of them were grouped under the BBAP reverse primer degeneration. In addition to the fact that Fra a 1 and Fra v 1 proteins and WRKY transcription factors are simultaneously acting defense-related proteins, knowledge is also emerging about their synergy. Peng et al. [26] summarized that PR3 and PR10 with other jasmonic acid synthesis-related genes LOX and AOS2 and overexpressing WRKY30 in the transgenic lines of rice increased endogenous jasmonic acid accumulation under the challenge of fungal pathogens what enhanced a whole resistance. Depression of PlWRKY65 expression resulted in significantly decreased PlPR2, PlPR4B, PlPR5, and PlPR10 expression in herbaceous peony [27]. CDDP marker technique was used to assess genetic diversity in 15 commercially important apple varieties where when used the same primer pairs as in this study, none of the apple varieties shared the same amplification pattern in all primer combinations taken together. The only concordant amplification profiles were generated for Red Delicious and Granny Smith (F1/R1), May Gold and Paula Red (F1/R2 and F1R3B), Selena and Melodie (F1/R2) but these cultivars differed in the remaining

profiles generated by other primer combinations [28].

4. Conclusions

The study was aimed at verifying BBAP and CDDP marker techniques as a useful tool to evaluate genetic diversity in strawberry genotypes. Both of the techniques proved to be convenient and suitable to generate polymorphic patterns in strawberry genotypes as none of the primer combination produced monomorphic profiles.

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