Assessment of genetic diversity of various components of pomegranate fruit with different skin color to SDS-PAGE electrophoresis method

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ABSTRACT

Pomegranate (Punica granatum L.) is one of the oldest and most important horticultural plants in Iran. Pomegranate is an important source of secondary metabolites such as phenolic compounds, tannins, colors and alkaloids. Since information about genetic diversity is a useful tool for planning experiments, and genetic improvement, in order to study the genetic diversity of various components of pomegranate fruit with different skin color, SDS-PAGE electrophoresis method was performed. Percentage of polymorphism in seed was 46/36 between genotypes and dendogram separated the whole tested genotypes into two main groups: the first cluster including Black genotype and second cluster including genotypes of Red, Green, White and Pink. Polymorphism among the five genotypes pomegranate peel was 58/82 percentage and cluster analysis separated the five cultivars of pomegranate into two distinct groups: the first group being Red and Black genotypes, and in the second group Green, White, and Pink. Percentage of polymorphism for juice was 48/14 and results of cluster analysis divided the five genotypes in two groups: the first group included the White genotype and the second group included Pink, Green, Red and Black genotypes. By observing protein bands in various components of pomegranate it could be concluded that protein can differentiate between the studied pomegranate species by producing some specific bands. Electrophoresis gel pattern of seed shows that the thickness of bands in seed organ is more than others, so this part of fruit can be useful for studying variation, in addition, the cluster analysis in pomegranate seed showed correlation between the intensity of color and total seed protein. Electrophoresis pattern of proteins of peel is somewhat similar to electrophoretic pattern of seed but the thickness of bands in peel was less than in seed. The result of protein bands in fruit peel shows that there is a relation between the color of pomegranate and the amount of protein because genotype with the same range color was put in the same group; furthermore, the pattern of gel electrophoresis shows that the resolution of protein bands in juice of pomegranate is low.

Key words: Genetic variation, pomegranate, SDS-PAGE, cluster analysis.

INTRODUCTION

Pomegranate (Punica granatum L.) is a deciduous shrub or small tree and is one of the oldest known edible fruits (Lansky and Newman, 2007). Pomegranate is included in the family of Punicaceae with 2n =2x =16, 18 (Smith, 1976) and over 1000 cultivars of P. granatum are extensively cultivated in many tropical and subtropical countries (Biale, 1981) such as Afghanistan, China, India, Iran, Japan, Mediterranean countries, Russia, and the USA (Lansky and Newman, 2007). There is growing interest in this fruit, not only for its taste but because modern science shows that
different parts of the fruit (peel, juices, seed and oil) contain strong antioxidant activity (Martinez et al., 2006; Lansky and Newman, 2007). Pomegranate is also an important source of secondary metabolites such as phenolic compounds, tannins, colors and alkaloid (Viuda-Martos et al., 2010; Wang et al., 2010).

Information about genetic diversity is a useful tool for parental selection in breeding, gen bank management, planning experiments, and genetic improvement (Ghafoor and Ahmad, 2005). Different methods such as morphological characterization, biochemical marker of protein (SDS-PAGE) and DNA level are applied for the assessment of genetic diversity for evaluation of germplasms (Khoshroo et al., 2013). Since the morphological traits are influenced by the environment, diversity studies through morphological traits are not reliable (Xue et al., 2006). On the other hand, genetic diversity and molecular studies on pomegranate using markers RAPD and AFLP, revealed that, despite the morphological diversity among pomegranate cultivars genetic differences are not large. Among different techniques used for assessment of genetic diversity, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) has become one of the most widely used techniques for a variety of plant proteins (Gepts, 1989). This method is fairly simple and economical, which are considered advantages for use in practical plant breeding (Rahman and Hirata, 2004). Also, storage proteins are largely independent of environmental fluctuation (Javid et al., 2004) and protein profiles are very specific and stable which supports the taxonomic, evolutionary relationships among crops (Ahmed et al., 2009).

The genetic variation of seed proteins in Date Palm cultivars was assayed by SDS-PAGE and the results showed that a total of 16 alternative protein bands with different mobility rates were identified within a molecular weight range of 11 to 350 KDa respectively (Khoshroo et al., 2013). Soluble proteins were extracted from leaves of sixty pear accessions and separated by polyacrylamide gel electrophoresis. Seven types of protein with 12 to 20 bands were observed. The accessions were classified into 12 groups and individual accessions varied from 0.71 to 0.97 similarity level (Ahmed et al., 2009). In 2008, a study was done to assess seed storage albumin in eight genotypes of Tunisian pomegranate, the results showed that pomegranate seed is rich in albumin. Protein pattern of SDS-PAGE were divided into three groups. Protein bands of the first group were in the range of 58 to 116 kDa molecular weight. Protein bands of the second group were from 33 to 46 kDa and for the third group between 15 and 23 kDa respectively (Elfallah et al., 2008).

This study was designed to investigate the genetic diversity of pomegranate genotypes using electrophoresis SDS-PAGE polymorphism between pomegranate genotypes with different skin colors that were assessed based on the protein of seed, peel and juice.

MATERIAL AND METHODS

Plant materials

Five Iranian pomegranate cultivars, including Poost Sefid Shirin (the sweet white skin), Poost Sabz Abarkoh (Abarkoh green skin), Poost Siyah Saveh (Saveh black skin), Poost Geermez Alak Saveh (Alak saveh light red skin) and Malas Poost Geermez (Malas red skin) grown in the agricultural research center of Yazd province, in the center of Iran, were used in this research. For each genotype, plant samples were collected randomly. All fruits were frozen in liquid nitrogen and stored at -80°C.

Preparation of protein samples

Seeds were isolated from fruit and passed over an air stream to remove the seed coats, then seeds, peels and juice of fruits were ground in liquid nitrogen in a mortar with pestle. About 1 g of different organs was suspended in 1 ml extraction buffer (buffer extraction: 0/5 M Tris-HCl at pH=8.8, 3% Glycerol, 2% SDS, 6.0 M urea and 5% β-mercaptoethanol). Homogenized mixture was incubated at 4°C overnight. Protein extraction buffer was properly mixed by vortexing for 2 to 3 min. The solubilized samples were centrifuged at 8000 rpm for 20 min at 4°C, and the supernatant saved and 100 ml of the extract mixed with 20 ml of loading dye (loading dye: A few drops of bromophenol blue, SDS 10% +1.5M Tris-Hcl, pH =8.8+ β-Mercaptoethanol + Glycerol) and stored at -20°C for further use.

Protein profiling using SDS-PAGE and gel electrophoresis

Sodium dodecyl sulphate – poly acryl amide gel electrophoresis (SDS-PAGE) was used to characterize the protein profiles of the accessions using 12.25% (w/v) separating gel and 5% (w/v) stacking gel (as developed by Laemmli (1970) with some modifications). To prepare 12.5% Resolving gel, Acrylamide 12.5%, SDS 1%, 375 mM Tris-Hcl, pH8.8, + APS 1/1000 v/v, TEMED 1/10000 v/v were combined and reached a volume of 100 ml with distilled water. Stacking gel (5%) was prepared with 5% Acrylamide, 250 mM Tris-Hcl, pH6.8, SDS 0.2%, APS 1/1000 v/v, TEMED 1/10000 v/v to final volume of 100 ml distilled water. Twenty micro liter of each sample was loaded on SDS-gel. A constant 200-150 mA electrical current was used for electrophoresis. Staining of the gels was done with Coomassie Brilliant Blue R-250 solution, containing 10% acetic acid, 45% methanol, and 45% water overnight. Gels were then destained by washing with a solution containing 10% acetic acid and 45% methanol, and 45% water until the color of background disappeared and electrophoresis bands were clearly visible. The protein bands were
visualized by transilluminator and photographs were taken for comparison of results.

Statistical analyses

Data from protein banding for different genotypes of pomegranate were displayed in different regions on polyacrylamide gels and the Relation Mobility (RM) of each band was calculated by determining the distance \( \text{RM} = \frac{\text{Distance migrated by the protein band from origin (cm)}}{\text{Distance migrated by tracking dye (cm)}} \).

All the monomorphic and polymorphic bands that were visible to the eye were scored and ambiguously scored bands were not used in the analyses. Depending upon the presence (1) or absence (0) of polypeptide bands, similarity index was calculated for all possible pairs of protein types. Presence and absence of bands were entered in a binary data matrix based on the results of electrophoresis band spectra. Data analyses were conducted using NTSys-pc, version 2.2 (Exeter software, Setauket, N.Y.). Similarities between cultivars were estimated using dice coefficient of similarity. Cluster analyses were conducted on similarity estimates using the unweighted pair-group method for arithmetic averages (UPGMA) and the resulting clusters were expressed as dendrograms (Sadia et al., 2009).

RESULTS

To avoid the stretch protein and single electrophoresis, the appropriate concentration of protein extracts was selected using the Bradford method. This made it possible to get clear bands. The number of main bands which were clear from different components of fruits ranged from 17 to 27. There were 19 clear bands in seed, 17 bands in peel and 27 bands in juice. Only high weight molecular bands were used for molecular analysis.

Seed

Figure 1 indicates profile of soluble proteins of Black, White, Red, Pink and Green genotypes. A total of 19 scorable protein bands was detected as a result of SDS-PAGE technique. Percentage of polymorphism was 46/36 between genotypes. The lightest weight molecular band had 56/25% relative mobility and the heaviest bands had 15/62% relative mobility, other bands were in the intervals between these two bands. The results of different protein bands pattern showed there are differences in relative mobility between different genotypes. Black genotype had band in relative mobility (62/15) and 75/68%, while same band did not appear in the other genotypes. The band number 3 with a relative mobility18/75% was observed in all genotypes except Red genotype. On the contrary, the band number 5 with relative mobility 23/75%was just observed in Red genotype. Protein band with relative mobility 31/25 was just in Pink genotype. Also, band with relative mobility 36/25 was not observed in Black and Green genotype.

According to the statistical analysis of the data in terms of the presence (1) and absence (0) of each band, cluster tree
showed that the two groups of plant species were separated from each other, four plant species are in one group, only one species in the group which was the Black genotypes. Furthermore, these 4 plant species formed two separate groups of one plant species and three plant species. The first sub-cluster including Red genotype, the second sub-clusters including Pink, White and Green genotypes (Figure 2a).

**Peel**

Polymorphism among the five genotypes pomegranate peel was 58/82. The highest number of bands was in White and Pink genotypes and the minimum number of bands was observed in Red and Black genotypes. While the loaded protein to all wells was equal, but density and intensity of different genotypes protein bands were not equal. Although, there were common protein bands in each genotype, some specific bands could be observed within each genotype (Figure 1). The bands number 5, 6, 9, 10, 11, 12, 15 with the relative mobilities of 28/12, 34/37, 52/5, 56/25, 58/12, 65/62, 75 and 84/37 respectively, were observed in all genotypes and the bands number 7, 8, 13, 14, 17 with the relative mobility of 43/75, 46/25, 75, 78/12 and 93/75 respectively, were present in all genotypes except Black and Red. Cluster analysis based on proteins separated the five cultivars of pomegranate into two distinct groups. Cultivars in the first group were Red and Black genotypes. The cultivars that appeared in the second group were Green, White, Pink, and the first sub-class contains ‘Green genotype and the second sub-class having two cultivars White and pink were identified respectively. Jacquard coefficient table confirms a cluster analysis (Figure 2b).

**Juice**

On the basis of the relative mobility of juice proteins on the gel, 27 bands were detected and used to explore genetic diversity (Figure 2). Highest number of bands was found in Pink genotype while minimum number of bands occurred in White. Percentage of polymorphism for all genotypes was 48/14. The band numbers 1 and 3, respectively, with relative mobility 12/5 and 87% were observed in all genotypes except White skin pomegranate. Similar results were obtained in bands number 4, 7, 14, 15, 21, 26 and 27. Band number 2 with relative mobility of 13/75 was just observed in White and Pink genotypes and the band number 11 was only observed in Pink genotype. Band with relative mobility of 71/87 in strip 22 was only observed in Pink and Red genotype. Results of cluster analysis divides five genotypes in two groups: the first group includes the White genotype and the second group includes Pink, Green, Red and Black genotypes. In the dendrogram of the cluster

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**Figure 2.** UPGMA dendrogram showing the relationships among 5 seed(a) and peel(b) Pomegranate accessions revealed by SDS-PAGE cluster analysis.
Figure 3. UPGMA dendrogram showing the relationships among 5 juice Pomegranate accessions revealed by SDS-PAGE cluster analysis.

analysis of proteins there are Pink genotypes in a sub-group and Red, Black and Green genotypes in a sub-group (Figure 4).

Conclusion

Different methods have been used for the analysis of genetic variations and identification of cultivars, among them protein electrophoresis is a good method because it is not affected by environmental fluctuation (Javid et al., 2004) and it is cheap and an easy method for distinctive genotyping (Javid et al., 2004; Iqbal et al., 2014).

It is the first report published about the use of seed, peel and extracts for protein studies in pomegranate. Proteins have been used as markers to estimate the genetic diversity in many crop species such as Iranian date palm, Ipomoea seed, Brassica species, etc (Khoshroo et al., 2013; Pragati et al., 2013; Sadia et al., 2009).

Although, the amount of protein loaded into all the wells was similar, density and intensity of bands were different in various parts of fruit. This study is able to demonstrate the difference in presence or absence of bands in different parts of fruit. Various amounts of protein in genes encoding proteins leads to making the difference in the abundance of bands in different organs of pomegranate Variation in the amount of protein has been observed in several studies such as some Onobrychis species growing in Turkey (Emre et al., 2007). Percentage of polymorphism between parts of fruit represents a variation between different studied species of pomegranate. All parts of different genotypes of pomegranate have common protein bands but a specific band was also observed in some genotypes. It could be concluded that protein can create a difference between the studied pomegranate species by producing some specific bands (NutanSinha et al., 2012).

Electrophoresis gel pattern of seed showed that the thickness of bands in seed organ is more than other parts and thicker bands have larger amounts of peptide strands such that this part of fruit can be useful for studying variation. In addition, the cluster analysis in pomegranate seed showed that correlation between the intensity of color and total seed protein because of genotype with light skin color (pink white and green ) was put in the same group and dark skin color genotypes (red and black ) were put into another group. As a result, it seems that cultivars
placed together in one group may have more similarity than the others (Kahrizi et al., 2013).

Electrophoresis pattern of proteins of peel in five cultivars (Figure 1) showed these cultivars are different in terms of diversity of protein bands and the results are somewhat similar to electrophoretic pattern of seed (Figure 4). However, the thickness of bands in peel were observed less than in seed, hence, it seems that the seed of pomegranate is more suitable for studying. Cluster analysis showed little variation between different pomegranate species (white, pink, green). Low variation was reported in different plant species such as sesame germplasm (Fazal et al., 2012) and chickpea (Ghafoor et al., 2003). The result of this part of fruit also showed that there is a relation between the color of pomegranate and the amount of protein because genotypes with same color range were put in the same group.

As mentioned earlier, just the white genotype was put in a separate cluster and other genotypes were put in the same group. The pattern of gel electrophoresis showed that the resolution of protein bands in juice of pomegranate is low. Since juice of pomegranate is full of anthocyanins and phenolic compounds (Gil et al., 2000), it seems that these compounds are more dominant than protein compounds in juice and this part of pomegranate cannot be a good option for studying protein variation.

In general, the results showed that there are differences in protein patterns in different parts of the fruit. The overall differences in the pattern of protein bands in different plants can be related to origin, evolution, and their genome (Khoshroo et al., 2013). The results indicated that the electrophoresis SDS-PAGE is an effective method for the separation of pomegranate genotypes, however, all variation in protein levels is not detectable; also just a group of the structural gene can order the production of proteins and this group may not reflect the overall genome, therefore, it is necessary to examine more number of genotypes to achieve more polymorphism and using of multiple polymorphism mechanisms will provide a better understanding of the relationship between genotypes (Ladizinsky and Hymowitz, 1979).

The overall degree of variation observed in pink, white and green genotypes was relatively moderate rather than black and red genotypes and as such can be suggested that the cultivars with similar banding patterns can be studied for detailed biochemical and molecular analyses including DNA markers and 2-D electrophoresis (Khoshroo et al., 2013). In addition, according to the importance of

Figure 4. Electrophoretic banding pattern of juice Pomegranate accessions generated by SDS-PAGE analysis.
pharmaceutical black genotypes (Moghaddam et al., 2013) must be noted as well as the specific profile of protein for this genotype; additionally, it is recommended that further studies must be done in order to investigate and more accurately in identifying the protein pattern of Black genotype and the role of this genotype to creating variation in diverse populations.

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