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Before-after analysis of genetic and epigenetic markers in garlic: A 13-year experiment

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ABSTRACT

Allium sativum is an important crop species in Argentina, one of the major garlic exporting countries in the world. Because garlic is an agamic propagated species, its breeding is based on the occurrence of plants with desirable traits, which are attributed to genetic mutations. In this work, we took advantage of a long term field experiment in a garlic line destined to genetic improvement to evaluate bulb weight and genetic and epigenetic stability and dynamics over 13 years. Average bulb weight increased from 65.3 g in the first year to 84.8 g the 13th year. Using AFLP (Amplified Fragment Length Polymorphism) and MSAP (Methylation Sensitive Amplified Polymorphism) techniques, we assessed genetic and epigenetic stability in plants of *A. sativum* taken at first, third and 13th year of field culture. We detected that 82.47 and 19.61% of the AFLP and MSAP fragments were present in the tree years of sampling. The application of selection pressure led to a reduction in genetic polymorphism and an increase of bulb weight, but did not influence epigenetic polymorphism, indicating that it is independent of genetic variability. We detected changes in the amount of each methylation pattern (unmethylated, hemimethylated and internally methylated) among the different years analyzed while 82.47 and 19.61% of the genetic and epigenetical loci were stable during the time of culture. Although some epiloci showed stability along the 13 years of culture, others presented gradual variation, while others were polymorphic within samples from the same year of culture. Finally, we discussed the implications of the high epigenetic variability of an agamic propagated species and its possible effect on the phenotype.

1. Introduction

Garlic (*Allium sativum* L.) is a widely cultivated crop species used as a spice and with medicinal purposes since ancient times. Argentina is one of the major garlic exporting countries and most of its production is carried out with monoclonal cultivars (García-Lampasona et al. 2012), which are expected to present no genetic variability given that all plants of a cultivar descend from a single plant. However, there is limited genetic variability due to mutations that enables garlic genetic improvement. This is a time consuming process because new monoclonal cultivars are obtained selecting plants/bulbs with distinguishable and better traits than the original population and the occurrence of differential traits is seldom (Burba 1993). Garlic breeding centers on the size of the bulb, as it is one of the fundamental characteristics for its commercialization.

It has been proved in different species that the phenotype is influenced by epigenetic marks (Cubas et al. 1999; Manning et al. 2006; Aversano et al. 2011; Dyachenko et al. 2014; Xia et al. 2016; Seymour and Becker 2017). In populations with low genetic variability, epigenetics can act as an important source of phenotypic variability (Róis et al. 2013). In addition, it is presumed that epigenetic contribution has higher potential in the adaptation of asexual species, since epigenetic differences accumulate easily (Verhoeven and Preite 2013). Knowing that garlic cultivars show wide phenotypic plasticity dependent on environmental factors–such as soil type, meteorological conditions, latitude, altitude, and cultural practices–(Volk et al., 2009; Hoogerheide et al. 2017) it is expected that epigenetic mechanisms exert some influence on garlic phenotype.

Many studies reporting genetic variability in garlic cultivars (Azuara Hernández et al. 2008; Chen et al. 2013; Chen et al. 2014; García Lampasona et al. 2003; Paredes et al. 2008; García-Lampasona et al. 2012; Gimenez et al. 2016; Manzum et al. 2014; Liu et al. 2015; Wang

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et al. 2016; Egea et al. 2017; Hoogerheide et al. 2017) proved that among various molecular markers AFLP is an excellent technique for detecting this variability when information on the genome is limited. It is known that garlic cultivars display a wide genetic, physiological and phenotypic variability; however, genetic variability has not been studied over time. In this sense, having a more comprehensive knowledge of the sources of genetic and epigenetic variation and the stability of markers over generations is crucial for germplasm utilization in garlic breeding. Here we took advantage of a long term field experiment in a garlic line destined for improvement and evaluated bulb weight and genetic and epigenetic status over 13 years of field culture with the goal of understand phenotypic, genetic and epigenetic stability over generations.

2. Material and methods

Plant materials used in this work come from a garlic genetic line destined for improvement. Plant cultivation was carried out at the Experimental Station La Consulta, INTA (National Institute of Agricultural Technology) 33° 42.7′ S and 69° 04.4′ W; 950 m.a.s.l. The experimental station is located in the Uco Valley, east of Los Andes mountains, and in an arid zone of Argentina characterized by low rainfall (200 mm per year), rigorous winters (-7 °C min – 25 °C max) and hot summers (7 °C min- 35 °C max).

Plants were grown in an alluvial soil, deep fine sandy loam Typic Torrifluvent (La Consulta series). This soil have values for medium total nitrogen content (709 mg/kg), medium to high for available phosphorus level (6 mg/kg), high exchangeable potash level (360 mg/kg), alkaline pH (7.8) and 1.25% organic matter. Clay content is about 8%; 30% is silt and 62% is sand, and almost 40% of sand was smaller than 250 µm. Water field capacity is 17% by weight, permanent wilting point is 9.4% by weight and bulk density is 1.35 kg/dm^3 (Lipinski and Gaviola 2011).

The crop was grown and cultured in the field according to the standard agronomic practices used by the growers at Experimental Station La Consulta INTA. In the mid-summer cloves weighing 4 g approximately were planted at 10 cm clove-clove distance in lines separated by 60 cm on field fertilized with animal manure at 1 kg/m2. The crop was irrigated every 10 days in winter and every five days in autumn and spring. Plants were harvested at the end of spring and were placed in a horizontal dryer. After 45 days, the bulbs were separated from the leaves, cleaned, and its weight was registered. Taking advantage of a long term experiment in which the weight of 100–300 bulbs was measured annually, we analyzed the data from years 1, 3, 5, 7, 10 and 13 with Kruskal Wallis using Infostat/P (Di Rienzo et al., 2014). Average minimum and maximum temperatures at EEA La Consulta were calculated for each month during the culture of garlic.

Fully expanded leaves of garlic plants were collected six months after cloves plantation in three different years named Y1, Y3 and Y13, corresponding to the first, third, and thirteenth year of culture. Three biological replicates were analyzed each year. DNA extraction was performed following Murray and Thompson (1980) protocol. The AFLP (Amplified Fragment Length Polymorphism) technique was performed following the general steps described by Vos et al. (1995) using six primer combinations (Suppl table). Only the reproducible amplification products from the triplicate reactions were scored. The MSAP (Methylation Sensitive Amplified Polymorphism) technique was conducted following the general steps of Xiong et al. (1999) using six primer combinations (Suppl table). Two isoschizomeric methylation-sensitive enzymes, *Hpa*II and *Msp*I, in combination with *EcoR*I were used to assess methylation in CCGG sequences. These enzymes have different sensitivity to certain methylation pattern of cytosines. *Hpa*II will not cut if either of the cytosines is fully (double-strand) methylated, but will cut if external cytosine is hemimethylated (single strand). While *Msp*I will not digest if the external cytosine is fully or hemi (single strand)-methylated, but it will digest if the internal cytosine is fully methylated. Based on the absence or presence of a band, cytosine methylation patterns were classified as unmethylated, hemimethylated, and internally methylated. AFLP and MSAP products were denatured at 90 °C in 4 µl of loading buffer, resolved by polyacrylamide gel (6%) electrophoresis at 85 W for 150 min, and visualized by silver staining.

Fragments from the AFLP and MSAP techniques were scored into a binary character matrix indicating presence (1) or absence (0). Only fragments within the 200–600 bp range were scored. A methylation status matrix was built from the *Hpa*II and *Msp*I datasets, being assigned into four categories according to the methylation pattern as follows: “1” when fragments are present in both *Hpa*II and *Msp*I (unmethylated sites); “2” fragments only present in *Hpa*II lane (hemimethylated CHG sites); “3” fragments only present in *Msp*I lane (full methylated CG sites) and “0” lack of fragment in both lanes (fully methylated 5´-CCGG sites or absence of the site) (Xu et al., 2004). The methylation status matrix was transformed into a methylation binary matrix, generating one line (or locus) for each methylation status and detailing only the presence (1) or absence (0) of the specific status. Based on MSAP data, number of methylated, hemimethylated, and unmethylated loci were determined for each year of culture. ANOVA was performed to compare the methylation patterns between years using Infostat software (Di Rienzo et al., 2014). For Venn diagram analysis we estimated the loci with fragment presence for each year (Y1, Y3 and Y13) and then performed pairwise comparisons to calculate the fragment shared by all three years, the fragments present in two years, and fragments exclusive from one year.

3. Results

Experimental Station INTA La Consulta has a garlic breeding program and an active germplasm bank that maintains cloves/seeds of different garlic cultivars obtained from several countries of the world. Over 13 years of evaluation, garlic plants traits showed progressive changes in size, clove and leaf number, and, particularly, in bulb weight (Fig. 1). In the 1st year, bulbs weighted 65 g in average and display a large dispersion with bulbs weighing between 40 and 82 g. The third year showed less dispersion and bulb weight sharply diminished to 47 g. In the fifth and seventh year the dispersion was reduced by a progressive increase in the average bulb weight to values of 82.2 g. By the 13th year the average weight increased to 84.8 g, and showed less dispersion (bulb size ranged between 76 and 102 g). The time in which bulbification begins depends, first, on plant exposure to low temperatures during the first months of culture (March-August) and then, on the

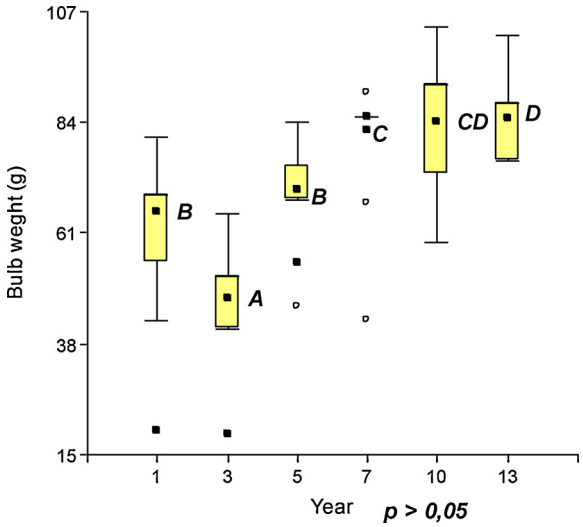

Fig. 1. Weight of bulbs harvested in year 1, 3, 5, 7, 10 and 13. Bars with the same letter are not significantly different.