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ORIGINAL ARTICLE

Assessment of genetic and epigenetic changes in virus-free garlic (*Allium sativum* L.) plants obtained by meristem culture followed by in vitro propagation

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Abstract

Key message **This is the first report assessing epigenetic variation in garlic. High genetic and epigenetic polymorphism during in vitro culture was detected. Sequencing of MSAP fragments revealed homology with ESTs.**

Abstract Garlic (*Allium sativum*) is a worldwide crop of economic importance susceptible to viral infections that can cause significant yield losses. Meristem tissue culture is the most employed method to sanitize elite cultivars. Often the virus-free garlic plants obtained are multiplied in vitro (micro propagation). However, it was reported that micro-propagation frequently produces somaclonal variation at the phenotypic level, which is an undesirable trait when breeders are seeking to maintain varietal stability. We employed amplification fragment length polymorphism and methylation sensitive amplified polymorphism (MSAP) methodologies to assess genetic and epigenetic modifications in two culture systems: virus-free plants obtained by meristem culture followed by in vitro multiplication and field culture. Our results suggest that garlic exhibits genetic and epigenetic polymorphism under field growing conditions. However, during in vitro culture

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system both kinds of polymorphisms intensify indicating that this system induces somaclonal variation. Furthermore, while genetic changes accumulated along the time of in vitro culture, epigenetic polymorphism reached the major variation at 6 months and then stabilize, being demethylation and CG methylation the principal conversions. Cloning and sequencing differentially methylated MSAP fragments allowed us to identify coding and unknown sequences of *A. sativum*, including sequences belonging to LTR Gypsy retrotransposons. Together, our results highlight that main changes occur in the initial 6 months of micro propagation. For the best of our knowledge, this is the first report on epigenetic assessment in garlic.

Keywords AFLP · MSAP · MSAP fragment cloning · Retrotransposon · Somaclonal variation

Introduction

Garlic (*Allium sativum* L.) is an important crop belonging to the family *Alliaceae*. Since the beginning of human history, it has been one of the earliest documented examples of plants employed for treatment of diseases and health maintenance (Rivlin 2001). Extensive research has been carried out on health promoting and medicinal properties of garlic and a diversity of biological activities were reported including antioxidant properties, cancer prevention, liver protection, immunomodulation and reduction of cardiovascular disease risk factors (Pittler and Ernst 2007; Iciek et al. 2009). Because of its multiple beneficial properties and worldwide changes in consumption habits, garlic global demand has risen considerably, and the garlic world production increased approximately ten million tons in the last ten years (FAO 2014).

At the same time, quality demands in garlic international markets are constantly increasing. According to this situation, the National Institute of Agricultural Technology (INTA, Argentina) developed several elite garlic cultivars. Among them, Perla INTA is of producer preference due to its high yield (22–25 t/ha), its suitability for fresh consumption and maintenance in cold chambers for 6 months (Burba 2003). Even though elite cultivars reach a high yield, one of the major problems widespread throughout all cultivation areas is the garlic mosaic viral disease, which perpetuates and accumulates from one generation to the next due to garlic vegetative propagation. To overcome this, virus-free garlic plants can be obtained by meristem culture in vitro, and several examples revealed an improvement in yield production after sanitization (Conci and Nome 1991; Conci et al. 2003; Lunello et al. 2007; Perotto et al. 2010).

In the production of virus-free garlic plants, the main purpose is to sanitize garlic plants and hence improve garlic quality, but it is also important to maintain the agronomic traits that define the elite cultivar (i.e., to grow plants morphologically and genetically identical to the mother plant). However, this improvement in plant sanity is frequently accompanied by somaclonal variation. Genetic variations observed in in vitro regenerated plants are largely stochastic, unpredictable, and non-reproducible (Larkin and Scowcroft 1981). Although somaclonal variation has been studied extensively, the underlying basis of this phenomenon remains unknown or at the level of theoretical speculation in crops. Recent studies attribute it to global epigenetic changes occurred by micro propagation in different species (Fraga et al. 2012; Huang et al. 2012; Rival et al. 2013).

The main objective of this work was to evaluate the effect of in vitro culture on genetic and epigenetic stability of *A. sativum* cv. Perla INTA plant lineages isolated from meristems and maintained through in vitro micro propagation culture over 6 and 12 months. Genetic and epigenetic data from these plants were compared with those from the first leaf previous to meristem culture and field-grown plants derived from the same garlic cultivar and polymorphisms were detected by AFLP and MSAP techniques. Cloning and sequencing of MSAP fragments with scored methylation status were carried out in order to determine their identity.

Materials and methods

Plant materials

Allium sativum cv. Perla INTA bulbs were provided by the Germplasm Bank of EEA INTA La Consulta, Mendoza, Argentina. This cultivar was registered in the National Institute of Seed from Argentina (INASE) in 1996. The crop was grown and maintained in the field since then according to the standard agronomic practices used by growers at EEA INTA La Consulta as follows: the fourth week of February cloves were planted on field fertilized with animal manure at 1 kg/m^2, in lines separated by 60 cm with a density of ten cloves per meter, irrigated every 10 days in winter and every 5 days in autumn and spring. Plant materials used in this work proceed from the original registered propagule of the cultivar.

Plants were grown under two different culture systems: field and virus-free plants obtained by meristem culture and then propagated in vitro (Suppl. Figure 1). In the first system, cloves were grown on field according to the agronomic practices described above. Leaf samples of three different plants (i.e., three replicates named FA, FB and FC) were

collected after 6 months of culture. For the second system, the first leaf of three garlic cloves was extracted, from which meristem was destined for in vitro regeneration and the remaining tissue was used for DNA extraction. Virus-free garlic explants were obtained by meristem culture. Garlic clove portions near the meristem, were cut into small cubes that contained the basal disc, sterilized in 70 % ethanol for 1 min and then in sodium hypochlorite solutions containing 0.5 % of active chlorine for 20 min. Meristematic domes with one leaf primordia (0.3 mm approximately) were excised and cultured in "D1" initiation medium (Conci et al. 1986). Cultures were kept at 21–23 °C under a photoperiod of 16 h light (52 μm s^{-1} m^{-2}). Developed plants were transferred to "D5" micro propagation medium (Moriconi et al. 1990). They were subcultured to fresh medium every 6–8 weeks. Plants were tested for virus presence by immunosorbent electron microscopy plus decoration (ISEM-D) according to Milne and Luisoni (1977). For trapping antisera at 1/1000 dilution in 0.05 M borate buffer pH 8.1 and for decoration 1/50 dilution of antisera were employed. Antisera against *Onion yellow dwarf virus, Leek yellow stripe virus, Garlic virus* C, *Garlic virus* A, and mix-antiserum produced in rabbit by injection with the mix of viruses that naturally affect garlic, taken From the stock of IPAVE-INTA, and obtained as described in Conci et al. (1999); Lunello et al. (2002) and Helguera et al. (1997) were used. *Garlic common latent virus* and *Garlic virus* D antisera were kindly provided by D. E. Lesemann, H. J. Vetten and E. Barg, BBA, Braunschweig, Germany; *Shallot latent virus* by L. Bos and D. Z. Maat, Research Institute for Plant Protection, Wageningen, Netherlands; *Garlic virus* C and *Garlic virus* A antisera by S. Sumi, Institute for Biotechnology Research, Wakunaga Pharmaceutical. Other antiserum, obtained from a mixture of garlic viruses, was kindly provided by M. Carvalho from UFV, Viçosa, Brazil, and R. Shepherd from the University of California, Davis (Carvalho 1981; Carvalho et al. 1981).

Three virus-free explants that were negative for virus analyses with all antisera described above were selected and maintained under in vitro conditions for 12 months. Samples were taken from the first leaf of garlic previous to meristem culture (named MA, MB and MC) and also at 6 (named IV6A, IV6B and IV6C) and 12 (named IV12A, IV12B and IV12C) months of in vitro culture. The first sample date for the micro propagated plants was determined at 6 months of culture when they presented enough vegetal material for virus analyses, DNA extraction and in vitro culture maintenance.

DNA extraction

DNA was isolated according to Murray and Thompson (1980) protocol with minor modifications. Briefly, frozen tissues (0.1 g) were ground in liquid nitrogen and 450 μl of extraction buffer (0.1 M Tris HCl, 0.5 M NaCl, 50 mM EDTA, 1.2 % SDS) were added to the resulting powder. The mix was incubated at 65 °C for 30 min. Then, 250 μl of chloroform:isoamyl alcohol (24:1) were added. The mix was centrifuged at 14,000 rpm for 15 min. Next, 5 μl of RNase (10 mg/ml) were incorporated to the resulting aqueous phase, incubated at 37 °C for 60 min and subsequently mixed with 250 μl of chloroform:isoamyl alcohol (24:1). The mix was centrifuged at 14000 rpm for 15 min. DNA was precipitated from the aqueous phase with 300 μl of ethanol (70 %). The pellet obtained was diluted in 100 μl water. Aliquots of 7 μl were used for spectrophotometric quantification (GeneQuant RNA/DNA Calculator, Pharmacia Biotech) and integrity was verified in 1 % agarose gel stained with ethidium bromide (0.5 mg/ml).

AFLP and MSAP analysis

The AFLP protocol was performed following Vos et al. (1995) with minor modifications. For the restriction reactions, 250 ng of DNA, 1.25 μl of buffer 2, 0.1 μl of *EcoRI* (10 U/μl), 0.2 μl of *MseI* (10 U/μl) and 0.1 μl BSA (100 μg/μl) in a 12.5 μl final volume were restricted at 37 °C for 3 h and enzymes were then inactivated at 65 °C for 20 min. Adapters ligation was performed with 6.25 μl restriction reaction, 1 μl *EcoRI* adapter (5 pmol/μl), 1 μl *MseI* adapter (50 pmol/μl) (Suppl. Table 1), 1 μl T4 ligase buffer (10×) (Promega), 0.4 μl T4 ligase (2 U/μl) (Promega) in a final volume of 12 μl, for 3 h at 20 °C and enzymes were then inactivated at 70 °C for 10 min. The ligation products were diluted 10 fold. Pre-amplification was conducted with 2.5 μl from the previous dilution, 2.5 μl PCR buffer (10×), 0.75 μl $MgCl_2$ (50 mM), 0.8 μl primer *EcoRI* +1 (50 ng/μl), 0.8 μl primer *MseI* +1 (50 ng/μl) (Suppl. Table 1), 1 μl of dNTPs (10 mM) and 0.2 μl Platinum Taq polymerase (5 U/μl), in a final volume of 20 μl. Reactions were carried out for 20 cycles of 30 s at 94 °C, 60 s at 56 °C and 60 s at 72 °C. Pre-amplification products were diluted threefold. Amplification was conducted with 5 μl of the diluted preamplification, 0.5 μl *EcoRI* +3 primer (50 ng/μl), 0.6 μl *MseI* +3 primer (50 ng/μl) (Suppl. Table 1), 0.4 μl dNTPs (10 mM), 2 μl PCR buffer (10×), 0.6 μl $MgCl_2$ (50 mM), 0.1 μl Platinum Taq polymerase (5 U/μl), in a final volume of 20 μl. The touchdown program consisted in 1 cycle of 30 s at 94 °C, 30 s at 65 °C and 60 s at 72 °C, decreasing the annealing temperature by 0.7 °C per cycle during 12 cycles, and 23 cycles for 30 s at 94 °C, 30 s at 56 °C and 60 s at 72 °C.

MSAP protocol was performed using the isoschizomers *HpaII* and *MspI* that recognize and digest 5′-CCGG sites, but they display differential sensitivity to DNA methylation (Suppl. Table 2). *HpaII* is inactive if one or both

internal and external cytosines are fully methylated (both strands methylated), but cleaves hemimethylated sequences (only a single DNA strand is methylated) and unmethylated sequences; whereas *MspI* digests sites with inner methylation of double-stranded DNA and unmethylated sites. MSAP was conducted following the general steps of Xiong et al. (1999), with minor modifications, as described below. For the restriction reaction, 6 µl of DNA (50 ng/µl), 4 µl of buffer (10×) and 1.5 µl of *EcoRI* (12 U/µl), were restricted at 37 °C for 3 h in a 40 µl final volume. Two digestion reactions were set up simultaneously for each sample, one for each methylation sensitive enzyme. The methylation sensitive restriction with *HpaII* enzyme was performed with 20 µl of *EcoRI* restriction reaction, 1.5 µl of *HpaII* (10 U/µl) and 4 µl of *HpaII* buffer (10×) in a 40 µl final volume. The methylation sensitive restriction with *MspI* enzyme was performed with 20 µl *EcoRI* restriction reaction, 0.75 µl *MspI* (20 U/µl) and 4 µl *MspI* buffer (10×) in a 40 µl final volume at 37 °C for 4 h and enzymes were then inactivated at 65 °C for 20 min. From here on, two parallel reactions were carried out for *EcoRI*/*HpaII* and *EcoRI*/*MspI* digestion products. Adapters ligation reactions were carried out with 20 µl restriction reaction, 2.5 µl *EcoRI* adapter (20 µM), 1 µl *MspI* adapter (50 pmol/µl) (Suppl. Table 1), 4 µl T4 ligase buffer (10×) (Promega), 0.4 µl T4 ligase (2 U/µl) (Promega) in a 40 µl final volume, for 3 h at 15 °C and enzymes were then inactivated at 70 °C for 10 min. Pre-selective amplification was conducted with 2 µl from the previous reaction, 2.5 µl PCR buffer (10×), 0.6 µl $MgCl_2$ (50 mM), 0.2 µl primer *EcoRI* +1 (20 µM), 0.2 µl primer *HpaII*/*MspI* +1 (20 µM) (Suppl. Table 1), 1 µl dNTPs (10 mM) and 0.2 µl Platinum Taq polymerase (5 U/µl), in a 20 µl volume. Pre-amplification products were diluted threefold. Amplification was conducted with 1 µl of the previous reaction, 0.2 µl *EcoRI* +3 primer (20 µM), 2 µl *HpaII*/*MspI* +3 primer (20 µM) (Suppl. Table 1), 1 µl dNTPs (10 mM), 2 µl PCR buffer (10×), 0.6 µl de $MgCl_2$ (50 mM), 2 µl Platinum Taq polymerase (5 U/µl), in a 20 µl final volume. The PCR programs for the pre-selective and selective amplifications were the same as in the AFLP protocol. 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.

AFLP and MSAP reactions of a subset of six samples were conducted by triplicate, starting from the same DNA extractions to calculate the error rate of the techniques. Loci with different patterns on replicates were excluded from the analyses as possible methodological artifacts. Enzymes and buffers for the restriction reactions were purchased from New England Biolabs whereas enzymes and buffers for PCR reactions were purchased from Invitrogen.

Data analysis

Fragments from AFLP and MSAP polyacrylamide gels 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 *HpaII* and *MspI* datasets (Suppl. Table 2), being assigned into four categories according to the methylation pattern as follows: "1" when fragments are present in both *HpaII* and *MspI* (unmethylated sites); "2" fragments only present in *HpaII* lane (hemimethylated CNG sites); "3" fragments only present in *MspI* 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 binary matrix, generating one line (or locus) for each methylation status and detailing only the presence (1) or absence (0) of the specific status.

We defined four sampling groups: field-grown plants (named F), leaves from cloves previous to meristem extraction (M), leaves from plants obtained by meristem culture and then micro propagated taken at 6 and 12 months of in vitro culture (named IV6 and IV12, respectively), and an analysis group denominated 'all data' which includes all the previously described groups.

A DNA methylation event was considered to be a polymorphism when at least one plant differed from the others in the methylation pattern within each sampling group. For global genetic and epigenetic analyses, polymorphic fragments obtained from AFLP and MSAP were counted and calculated as a percentage of polymorphic fragments within a group using FAMD software (Schlüter and Harris 2006). Dice coefficient and dendrograms derived from UPGMA cluster analysis were performed using NTSYSpc 2.01e (Rohlf 1998). Bootstrap analysis was conducted with WinBoot (Yap and Nelson 1996). The number and percentage of 5′-CCGG sites with each of three methylation patterns presented in Suppl. Table 2 were determined for each group.

Pair comparisons among individuals of different groups (M–F, M–IV6, M–IV12 and IV6–IV12) were performed, from which levels of genetic and epigenetic loci-specific changes were estimated. For genetic analysis, we considered the sum of fragments gain and loss along those comparisons as changes. For epigenetic analysis, we classified methylation status progression in four categories described in Suppl. Table 3: unchanged methylation patterns (fragments that exhibited the same methylation status in one individual among the different analyzed stages), patterns turned into unmethylated sites (demethylation); patterns turned into CG methylated sites (methylation), and patterns turned into CNG hemimethylated sites. For each case, the number of events was recorded and plotted in a

box-plot. ANOVA was performed and in the case of significant results, mean comparisons by LSD were conducted using INFOSTAT 2014 version (Di Rienzo et al. 2014).

Cloning and sequencing of differentially methylated MSAP fragments

To investigate the identity of the sequences that altered their methylation status, we extracted and sequenced MSAP fragments that constitute methylation targets during in vitro culture. Fragments were excised from the polyacrylamide gel and hydrated in 20 µl milliQ water through incubation at 95 °C for 20 min. Eluted DNAs were used as templates to amplify by PCR reactions using the appropriate primers. Sizes of PCR products were verified by agarose gel electrophoresis and extracted from the gel before ligation into plasmid vector pGEM-T Easy (Promega) and transformed into TOP10 chemically competent *Escherichia coli* cells. The plasmid DNA of individual clones was obtained by the alkaline lysis procedure (Kotchoni et al. 2003) and digested with *EcoRI* enzyme to verify the presence of insert. Single clones positive for inserts were selected and sequenced using the M13 forward primer in an ABI PRISM 3100 (Applied Biosystems) genetic analyzer. All nucleic acid sequences were screened for vector contamination using the Vector Screen program (http://www.ncbi.nlm.nih.gov/VecScreen) and primer sequences were removed. Homology search was conducted using the BLAST program in different databases of the GenBank (BLASTN: nt, est, wgs, gss and HGTS; BLASTX: pdb and nr; and search for conserved domains in cds) of the National Center of Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov). Homology assignation criterion was based on maximum probability threshold per sequence and E value $<10^{-15}$.

Results

Global genetic and epigenetic analyses

For the AFLP analysis, five primer combinations yielded 379 scorable fragments, 55.9 % of which were polymorphic in all data set (Table 1). In vitro cultured explants showed higher polymorphism within group than meristem and field-grown samples. A comparison between different times of sampling (Suppl. Figure 1; M, F, IV6 and IV12) in the two culture systems revealed a 2.9-fold increase in genetic polymorphism from M to F (from 2.4 to 6.9 %), an 8.1-fold increase from M to IV6 (from 2.4 to 19.3 %) and a 12.9 fold increase from M to IV12 (from 2.4 to 30.6 %). The minimum accumulation of polymorphism was given from IV6 to IV12, with 1.6 fold increase (from 19.3 to 30.6 %).

Table 1 Number and percentage (in parentheses) of polymorphic fragments detected in AFLP and MSAP binary matrices of garlic plants (cv. Perla INTA) grown under field conditions (F) meristem (M), and plants micropropagated in vitro during 6 (IV6) and 12 (IV12) months

Plant group	AFLP	MSAP
All data	212 (55.93)	298 (99.00)
F	26 (6.86)	92 (30.56)
M	9 (2.37)	83 (27.57)
IV6	73 (19.26)	73 (24.25)
IV12	116 (30.60)	107 (35.54)

For the MSAP analysis, three primer combinations for selective amplifications were chosen among the ten primer combinations for providing the most reliable and consistently scorable fragments. These primer combinations generated 145 scorable fragments that were used to construct a methylation status matrix, which was then transformed into a binary matrix. The latter returned 301 methylation loci, 99 % of which were polymorphic for all data set (Table 1). Epigenetic polymorphisms were higher than genetic polymorphisms in every level analyzed; however, differences in epigenetic polymorphism among sampling groups were smaller than genetic one. In this sense, whereas the increase level in epigenetic polymorphism was 1.1 fold in M–F comparison, a slight decrease of 0.8 fold occurred in M–IV6 and values of 1.3 and 1.5 fold increase were observed in M–IV12 and IV6–IV12, respectively. At 12 months of in vitro culture we detected the highest epigenetic polymorphism (35.55 %).

The genetic and epigenetic relationships among plants were represented in dendrograms obtained from Dice similarity coefficients (Fig. 1). In the case of AFLP markers, the genetic similarity ranged from 0.776 (among IV6B and IV12A) to 0.993 (MA and MB). The genetically closest samples were grouped in the superior branch (Fig. 1a), in which the three M samples (A, B and C) exhibited high genetic similarity (higher than 0.99), F samples (A, B and C) joined the cluster (genetic similarity coefficients range from 0.97 to 0.99). On the other hand, the three explants lineages (A, B and C) exhibited lower similarity at IV6 (range 0.89–0.94) and IV12 (0.81–0.91) than M samples.

Comparably to epigenetic polymorphism, Dice similarity coefficients for MSAP markers (Fig. 1b) showed higher variability than genetic coefficients, ranging from 0.141 (IV12C and MB) to 0.851 (IV6A and IV6B). The samples were grouped in two principal clusters that separated in vitro cultured explants (lower branch) from F and M samples (upper branch). In the latter group, M and F were disposed in two different subgroups whereas, in the lower branch, group limits between IV6 and IV12 were less clear.

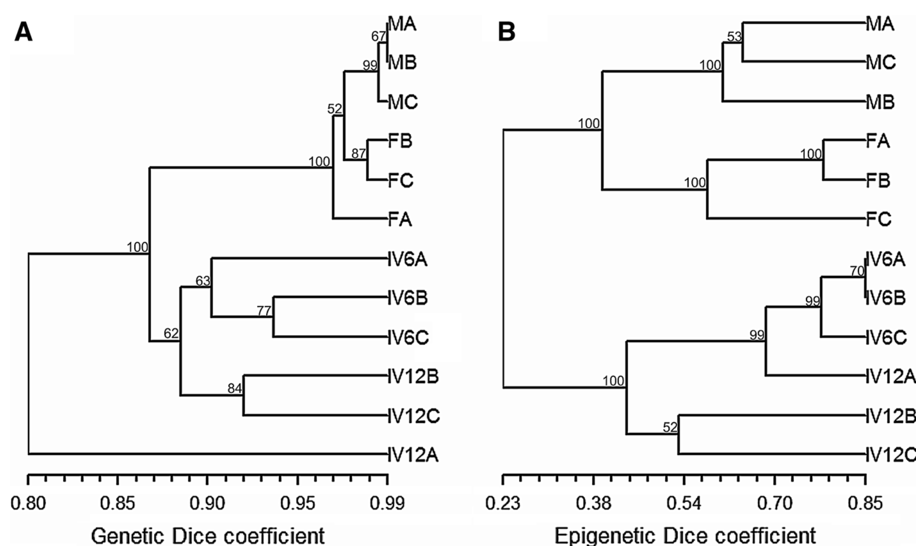

Fig. 1 UPGMA cluster analysis based on Dice similarity coefficients calculated from (**a**) AFLP and (**b**) MSAP data of garlic plants (cv. Perla INTA) grown under field conditions (F), meristem (M), and plants micro propagated in vitro during 6 (IV6) and 12 (IV12) months

Regarding the methylation patterns, M exhibited the three methylation patterns almost equally represented (Table 2), while in the remaining sampling groups the hemimethylated pattern was the least abundant. Comparisons of the abundance of the methylation patterns among M to F revealed a slight increase of 1.3 fold in unmethylated and CG methylated sites. The most important differences were detected under in vitro culture conditions, mainly from M to IV6, with a 2.7-fold significant increase in CG methylated sites and a 1.8-fold increase in unmethylated sites; whereas CNG hemimethylated sites exhibited a decrease trend. On the contrary, from IV6 to IV12 CG methylated and unmethylated sites showed a decrease in abundance, reaching values similar to those found in M.

Loci-specific genetic and epigenetic changes

We compared AFLP fragment presence and alterations in epigenetic patterns among sampling groups: M–F, M–IV6, M–IV12 and IV6–IV12 (Fig. 2). At the genetic level, a few changes were detected from M to F, while a progressive increase of changes were observed during in vitro culture (M–IV6 and IV6–IV12), with the highest number of changes accumulated in M–IV12 (Fig. 2a). On the other hand, at the epigenetic level, the majority of the changes were observed in M–IV6, while sites turning into unmethylated (Fig. 2d) and CG methylated (Fig. 2e) were significantly higher than M–F and IV6–IV12. In the last 6 months of in vitro micro propagation (IV6–IV12), few changes were observed. In M–IV12 the number of sites turning into unmethylated (Fig. 2d) and CG methylation (Fig. 2e) were intermediate between M–IV6 and IV6–IV12. M–F and IV6–IV12 presented the greatest amount of sites that did not change their methylation pattern (Fig. 2c). Conversely, the number of MSAP patterns that turned into CNG methylated sites was similar in all the comparisons (Fig. 2f).

Cloning and sequencing of differentially methylated DNA sequences

Forty-six MSAP DNA fragments were isolated, sequenced and submitted to DDBJ database (IDs AB937728–AB937773). All sequences presented the CCGG site. Fourteen sequenced fragments returned BLAST results

Table 2 Number of cytosine methylation levels analyzed through MSAP of garlic plants (cv. Perla INTA) grown under field conditions (F) meristem (M), and plants micropagated in vitro during 6 (IV6) and 12 (IV12) months

Plant group	Unmethylated CCGG sites	CNG hemimethylated	CG methylated
F	30.0 ± 6.0	23.3 ± 4.8	33.0 ± 2.0^{b}
M	23.7 ± 1.3	24.3 ± 1.9	24.3 ± 3.8^{b}
IV6	44.0 ± 2.1	11.3 ± 1.7	67.3 ± 5.0^{a}
IV12	30.0 ± 5.8	17.0 ± 4.7	25.3 ± 7.9^{b}
$F_{(11,2)}$	3.91 (ns)	2.84 (ns)	15.54*

Average data (±SE; $n = 3$) with the same letter within a column are not significantly different (LSD test, * $p < 0.01$)

ns not significant

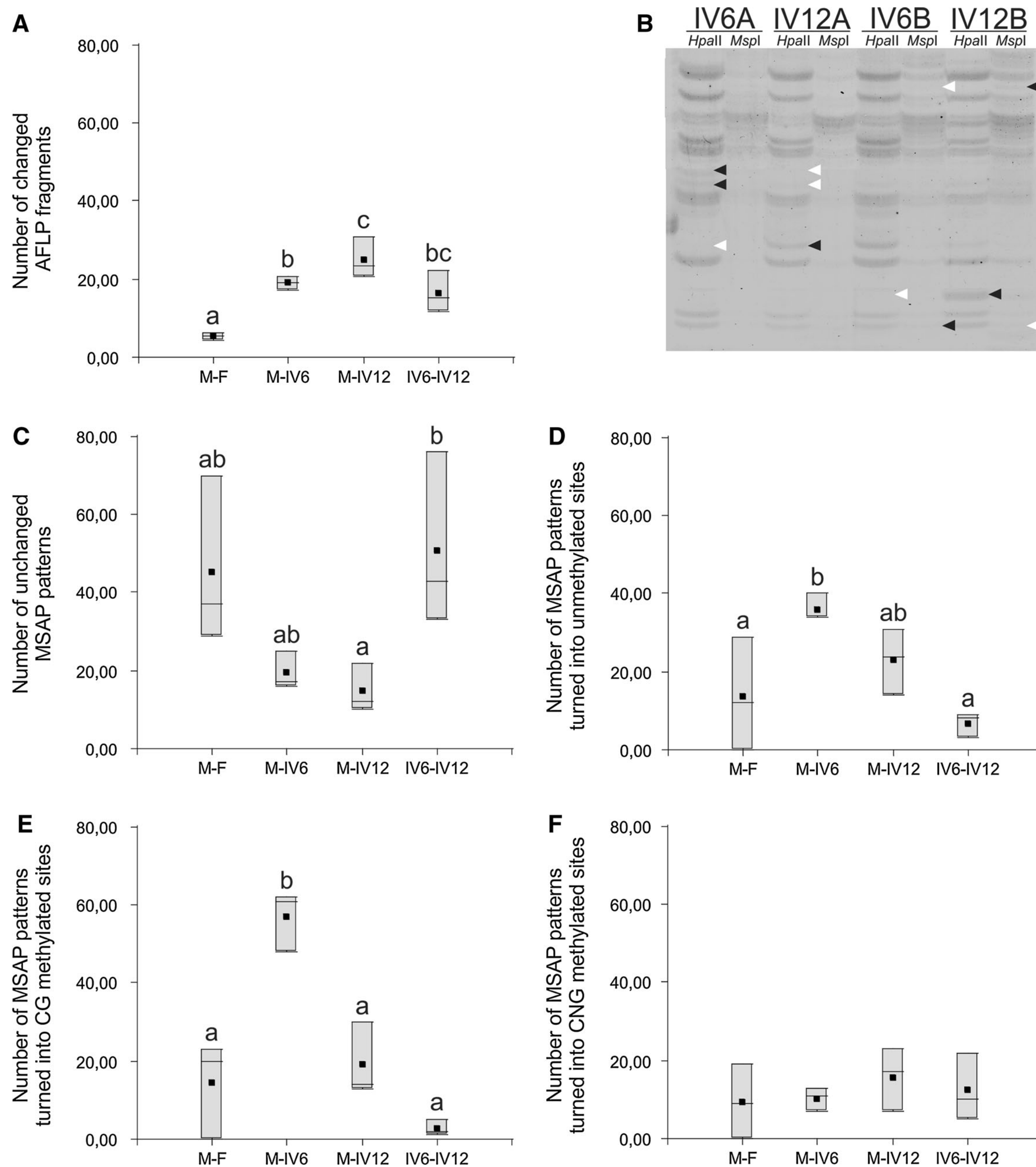

Fig. 2 Loci-specific genetic and epigenetic changes among sampling times: meristem (M), field-grown plants (F) and in vitro cultured explants at 6 (IV6) and 12 (IV12) months. **a** Loci-specific genetic changes. **b** MSAP polyacrylamide gel showing alterations in methylation patterns indicated by *black* (presence) and *white arrows* (absence). **c** Number of epigenetically stable loci. **d** Number of methylation changes turned into unmethylated pattern. **e** Number of methylation changes turned into CG methylated pattern. **f** Number of methylation changes turned into CNG methylated pattern. Data are represented in a box-plot diagram. Median ± IQD ($n = 3$); (*closed square*) inside the *box* indicates arithmetic mean. *Boxes with the same letter* within a graphic are not significantly different with LSD test (for **a**, **c** and **d** graphics, $p < 0.01$; in **e** graph, $p < 0.05$ and in **f**, non-significant differences was identified)

with significant similarity to annotated sequences (Table 3). One MSAP fragment showed significant similarity to a 5S ribosomal RNA gene. Possible functions could be assigned to four others sequences: a threonine synthase and three conserved domains from putative retrotransposons proteins (integrase, reverse transcriptase and RNase H like).

Discussion

Garlic plants are systemically infected by many viruses. Because this species is propagated only in an agamic way, viruses accumulate in bulbs and perpetuate the infection from one generation to the next (Shahraeen et al. 2008; Conci 1997; Perotto et al. 2010; Conci et al. 2010; Taşkin

Table 3 BLAST results of 14 differentially methylated DNA fragments obtained from MSAP of in vitro cultured garlic plants, cv. Perla INTA

MSAP fragments		Sequence homology				
Accession number	Size	Score	*E* value	Accession number	Database	Description
AB937769	333	553	5.00E−154	JF496591	EST (BLASTN)	5S ribosomal RNA gene region *A. sativum*
AB937763	236	326	4.00E−86	GAAO01011675	EST (BLASTN)	mRNA *A. cepa*
AB937762	362	262	1.00E−67	FS218499	EST (BLASTN)	mRNA *A. sativum*
AB937746	301	197	3.00E−47	FS221676	EST (BLASTN)	mRNA *A. sativum*
AB937753	254	188	1.00E−45	FS211644	EST (BLASTN)	mRNA *A. sativum*
AB937749	336	188	2.00E−45	FS211644	EST (BLASTN)	mRNA *A. sativum*
AB937764	244	176	7.00E−41	FS211644	EST (BLASTN)	mRNA *A. sativum*
AB937730	141	127	6.00E−27	FS214333	EST (BLASTN)	mRNA *A. sativum*
AB937740	315	129	8.00E−27	GAAN01027809	EST (BLASTN)	mRNA *A. cepa*
AB937746	301	133	8.00E−28	FS221676	EST (BLASTN)	mRNA *A. sativum*[a]
AB937734	177	122	3.00E−25	FS214333	EST (BLASTN)	mRNA *A. sativum*
AB937761	233	95.5	7.00E−23	XP_006665068	nr (BLASTX)	Uncharacterized protein *Oryza brachyantha*[b]
AB937765	304	74.7	4.00E−15	ABF98364	nr (BLASTX)	Putative retrotransposon protein *Oryza sativa*[c]
AB937736	139	86.0	5.00E−15	DQ341437	EST (BLASTN)	mRNA similar to threoninesynthase *Triticum aestivum*[d]

[a] Conserved domain: integrase Ty3-gypsy retrotransposon

[b] Conserved domain: reverse transcriptases (RTs) from retrotransposons and retroviruses

[c] Conserved domain: RNase H like

[d] Conserved domain: threoninesynthase

et al. 2013). The most common method for obtaining virus-free garlic plants is the meristem culture, which combined with thermotherapy has been frequently used to increase the possibilities of success in removing viruses (Perotto et al. 2003; Conci et al. 2010; Taşkin et al. 2013). However, it is known that thermotherapy has deleterious effects on plant regeneration compared to meristem culture alone (Ramírez-Malagón et al. 2006) and garlic virus-free plants can be obtained without employing thermotherapy (Conci and Nome, 1991; Perotto et al. 2010; Conci et al. 2005; Taşkin et al. 2013). In this study we obtained virus-free garlic plants throughout meristem regeneration in vitro, avoiding thermotherapy and thereby eluding a possible source of somaclonal variation.

Regarding the multiplication of elite cultivars of virus-free garlic plants for commercial production, it is important to evaluate genetic and epigenetic stability along the process because low rates of multiplication in vitro make this process time-consuming (to obtain enough garlic plants) and various crop cycles of multiplication in the field after sanitization process are necessary (to attain a large number of virus-free garlic propagules). On the other hand, it has been reported that garlic in vitro culture generates somaclonal variation (Al-Zahim et al. 1999; Burba personal communication). Hence, it is important to determine critical points of this system that can affect the stability of the cultivar and to compare it with traditional field growth systems. Taking these considerations into account, in this study we analyzed the genetic and epigenetic consequences of an in vitro method to sanitize and micro propagate elite cultivars of *A. sativum*.

Asexual propagated cloves of Perla INTA are genetic and epigenetically instable

Our results suggest that, although low, there is intra-cultivar genetic variation in Perla INTA, and that the level of genetic polymorphism is related to the type of growth system (Table 1; Fig. 2a). In this sense, while the estimated genetic variability of field-grown Perla INTA is around 4 %, during in vitro propagation of the plants obtained by meristem culture it can reach values of 15 % (Fig. 1a). It is important to highlight that studies carried out in garlic assume that cultivars are clones, hence genetically invariable and without intra-cultivar genetic variation. However, Volk et al. (2004) detected genetic polymorphism at intra-cultivar level in different accessions of garlic. This phenomenon was also observed in ornamental and asexual

species belonging to the genus *Allium* (Krzymińska et al. 2008), and also in other monocotyledonous agamic species such as *Agave tequilana* (Torres-Morán et al. 2010) and *Ananas comosus* (Santos et al. 2008). Genetic diversity within garlic populations might be due to mutations that accumulated over time owing to non-reduction in grower's selection pressure (Simon and Jenderek 2003) or to multiclonal composition of the cultivar under analyses (Krzymińska et al. 2008). However, considering the monoclonal origin of Perla INTA accession and the low frequency of genetic polymorphism detected in the initial garlic clove pool, it is most probable that mutations are responsible for this variation. In any case, more research is necessary to unveil the molecular mechanisms underlying this phenomenon.

On the other hand, unlike the results obtained from genetic analyses, we observed high levels of epigenetic variation within the analyzed samples, and a specific epigenetic differentiation according to the growth system employed (Table 1; Fig. 1b). DNA methylation is involved in several cell processes from gene regulation to maintenance of heterochromatin architecture and it is implicated in a wide variety of processes from ontogenic development to abiotic stress responses (Piccolo and Fisher 2014). Although there is no information about methylation status in *A. sativum* genome, it is known that the genome of the garlic relative *A. cepa* (onion) is under high epigenetic control, with CG dinucleotides distributed in entire chromosomes and elevated levels of heterochromatin highly methylated (Suzuki et al. 2001). Despite differences in reproductive strategies between onion and garlic, and considering genome size and organization similarities, it is possible to predict related regulation mechanisms occurring in garlic.

In vitro culture of Perla INTA induces genetic and epigenetic changes

In vitro culture is considered to be a stressful environment for plant tissue, leading to mutation and the generation of polymorphism (Kaeppler and Phillips 1993; Phillips et al. 1994). Our results on in vitro propagation of the plants obtained by meristem culture suggest that genetic polymorphism increases progressively in the course of in vitro culture in Perla INTA, reaching values of 30 %, whereas epigenetic polymorphism was maintained at high values during all the stages (Table 1). Several reports compare genetic and epigenetic changes induced by in vitro propagation of the plants, with variable and contrasting results. Similar results were observed in plants obtained by somatic embryogenesis in *Theobroma cacao* (Rodríguez López et al. 2010) and *Freesia hybrida* (Gao et al. 2009) or nodal tissue-culture in *Solanum tuberosum* (Dann and Wilson 2011). Regarding meristem in vitro culture, while some reports suggested that it induces high epigenetic polymorphism and scarce or null genetic polymorphism (Wu et al. 2011), others revealed null polymorphism at both levels (Bobadilla Landey et al. 2013). However, the use of different protocols of in vitro culture and different plant materials makes reaching definitive conclusions very difficult.

The comparison among sampling times enables us to detect the genetic and epigenetic variation produced and to propose environmental change as a possible cause for these variations. For instance, M–IV6 enables the detection of changes related to in vitro establishment; IV6–IV12, the specific variation related to the time of culture and M–IV12, the time-accumulated variation in the whole process of in vitro regeneration and micro propagation. With this analysis, we can suggest that in Perla INTA, the amount of genetic changes produced in the first and last 6 months of in vitro culture were similar (Fig. 2a), while most epigenetic changes were produced in the first half of culture and decreased during the last months, taking values similar to those found in M–F (Fig. 2d, e).

Changes in DNA methylation are a usual response under different in vitro culture systems in different plant species (Us-Camas et al. 2014). It appears that there is not an epigenetic universal trend in plant genomes under in vitro culture since high levels of CG methylation has been reported (Smulders et al. 1995; Wang et al. 2012), as well as a decrease methylation trend (Kaeppler and Phillips 1993; Li et al. 2007; Gao et al. 2009). For Perla INTA in vitro cultured plants, during the first 6 months of in vitro culture there is an increase of CG methylated and unmethylated sites (Table 2) and a decrease in the following 6 months, reaching similar values to those found in the original material used for in vitro culture (M).

Another interesting results are the similar and low values of hemimethylation (CNG sites) changes during all stages analyzed. In agreement with our results, several studies reported that the hemimethylated pattern is the least abundant, (Gao et al. 2009; Díaz-Martínez et al. 2012; Wang et al. 2012) possibly indicating that this state is transient and intermediate to full methylation or demethylation of both strands.

Several reports suggested that prolonged in vitro culture contributes to accumulation of genetic (Li et al. 2007; Peredo et al. 2009; Baránek et al. 2010) and epigenetic changes (Kaeppler et al. 2000; Sun et al. 2013); therefore, limiting time of in vitro culture could minimize somaclonal variation. Furthermore, modifying culture conditions is another strategy employed to overcome somaclonal variation (Bobadilla Landey et al. 2013). Our future efforts will focus on reducing this variation during the initial months of in vitro culture in Perla INTA.

Sequence analysis of MSAP fragments

Despite the availability of complete genome sequences and a high density of EST and protein libraries of different monocotyledons (Yu et al. 2002; Paterson et al. 2009; Brenchley et al. 2012), about 60 % of the sequenced fragments showed no significant similarity to annotated sequences. This might be attributed to the fact that most sequenced monocotyledon genomes belong to the family Poaceae, which presented scarce or null synteny with *Allium spp*. (Jakše et al. 2008). Nevertheless, a complete genome sequencing project of *A. cepa* and EST sequencing projects of both *A. sativum* and *A. cepa* are ongoing (Kim et al. 2009; McCallum et al. 2012; Sun et al. 2012). These advances will allow us to understand the genetic and epigenetic mechanisms of this genus in more depth.

Regarding the identity of the DNA fragments affected by epigenetic changes, our sequencing results indicate that 30 % of the MSAP fragments correspond to coding regions (Table 3). This percentage is considered high, taking into account that garlic probably has a low gene density as observed in the *A. cepa* genome (Jakše et al. 2008) and it might indicate epigenetic regulation of gene expression during in vitro propagation of the plants obtained by meristem culture.

It has been reported that certain genes are specifically regulated by DNA methylation during in vitro culture (De-la-Peña et al. 2012; Wang et al. 2013). The target genes of this regulation are related to several cellular processes such as metabolism, cellular signals, transcription, cell division, and protein metabolism (Us-Camas et al. 2014). However, in this work we could only assign a putative function to five sequences that exhibited similarity to rRNA 5S, a threonine synthase and three proteins belonging to Gypsy superfamily of LTR retro elements.

Nuclear ribosomal DNA (rDNA) encoding 5S rRNA is one of the most important housekeeping genes playing a central role in cell metabolism. In plant genomes several hundreds and up to tens of thousands of highly homogeneous copies of 5S rRNA gene are organized as separate clusters of tandem repeats (Douet and Tourmente 2007; Garcia et al. 2012). However, not all tandems are transcriptionally active and it has been shown that are highly methylated in different plant species (Mascia et al. 1981; Goldsbrough et al. 1982; Rafalski et al. 1982; Grellet and Penon 1984; Ellis et al. 1988; Fulnecek et al. 1998). In the model plant *Arabidopsis thaliana*, methylation-dependent and methylation-independent silencing pathways of 5S rDNA tandem repeats have been identified (Douet and Tourmente 2007).

The enzyme threonine synthase (TS), together with cystathionine-γ-synthase (CGS), operate in concert to regulate carbon flow towards methionine in plants. The competence for substrate (*O*-phospho homoserine) between TS and CGS is finely regulated by post-transcriptional and post-translational mechanisms (Amir et al. 2002). Therefore, regulation by methylation of the TS gene could implicate the control of threonine biosynthesis, in the TS pathway, or methionine and *S*-adenosylmethionine (SAM) biosynthesis in the CGS pathway. Furthermore, SAM is the primary biological methyl-group donor in plant metabolism; then, this regulation has influence in many other cellular processes, including DNA methylation.

Plant retro elements are the major constituents of plant genomes, and their mobility is closely monitored and regulated by the host genome, mainly by epigenetic control (Chandler and Walbot 1986; Fedoroff 2012). In fact, high correlation between transposons mobility and methylation status in the vicinity of retro elements was described in different species (Kashkush and Yaakov 2011; Paz et al. 2015), including under in vitro culture conditions (Ngezahayo et al. 2009).

There is limited information concerning the activity of retro elements in *Allium* spp. genomes. However, previous reports suggested that intact and recently inserted LTR retrotransposons are the major components of *A. cepa* genome with families representing the 3–4 % of the entire genome (Vitte et al. 2013). Nevertheless, Jakše et al. (2008) observed that different retro element populations expanded in *A. sativum* and *A. cepa*. The putative retro element sequences described in this work were amplified from cDNA obtained from meristem culture of different cultivars of *A. sativum* (Yañez Santos AM, Gimenez MD, Paz RC and García Lampasona SC, unpublished results), suggesting that they might be active in this genome. However, more research is required to validate the identity and role of these sequences in somaclonal variation in garlic.

In summary, our results suggest that there is certain level of genetic and epigenetic variation in garlic under standard growing conditions. These changes are more dramatic during in vitro propagation of plants obtained by meristem culture. In this sense, while genetic polymorphism increases along different stages of in vitro culture, the epigenetic polymorphism undergoes a smaller variation. However, when analyzing methylation patterns separately, most changes occur during the first 6 months with significantly increased levels of CG methylation and 5′-CCGG site demethylation, whereas during the following 6 months, there is a slight tendency to revert to the initial epigenetic state. Moreover, the fact that about a third of the sequenced MSAP fragments presented similarity with ESTs indicates that the gene expression might be regulated by DNA methylation during in vitro culture. Particularly, the detection of methylation variation in genes coding 5S rRNA and threonine synthase indicates that methylation

changes during in vitro culture exert control on cellular metabolism. On the other hand, the identification of retro elements among MSAP fragments suggest that their activity could be regulated epigenetically and that their epigenetic activation might be one of the mechanisms by which genetic variability accumulates during in vitro culture.

Author contribution statement The authors performed the following contributions to the investigation presented in this manuscript: MD Gimenez, DNA extraction, AFLP and MSAP reactions and analysis, manuscript redaction; AM Yañez Santos, cloning and sequencing of MSAP fragments; RC Paz, cloning and sequencing analysis and manuscript redaction; MP Quiroga, in vitro culture, DNA extraction and MSAP reactions; CF Marfil, MSAP advice; VC Conci, in vitro culture, virus analysis and work direction; SC García Lampasona, work direction.

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Compliance with ethical standards

Conflict of interest Authors declare that they have no conflict of interest.

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