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Morphological features of different polyploids for adaptation and molecular characterization of CC-NBS-LRR and LEA gene families in *Agave* L.

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ABSTRACT

Polyploidy has been widely described in many *Agave* L. species, but its influence on environmental response to stress is still unknown. With the objective of knowing the morphological adaptations and regulation responses of genes related to biotic (LEA) and abiotic (NBS-LRR) stress in species of *Agave* with different levels of ploidy, and how these factors contribute to major response of *Agave* against environmental stresses, we analyzed 16 morphological trials on five accessions of three species (*Agave tequilana* Weber, *Agave angustifolia* Haw. and *Agave fourcroydes* Lem.) with different ploidy levels ($2n=2x=60$ $2n=3x=90$, $2n=5x=150$, $2n=6x=180$) and evaluated the expression of NBS-LRR and LEA genes regulated by biotic and abiotic stress. It was possible to associate some morphological traits (spines, nuclei, and stomata) to ploidy level. The genetic characterization of stress-related genes NBS-LRR induced by pathogenic infection and LEA by heat or saline stresses indicated that amino acid sequence analysis in these genes showed more substitutions in higher ploidy level accessions of *A. fourcroydes* Lem. 'Sac Ki' ($2n=5x=150$) and *A. angustifolia* Haw. 'Chelem Ki' ($2n=6x=180$), and a higher LEA and NBS-LRR representativeness when compared to their diploid and triploid counterparts. In all studied *Agave* accessions expression of LEA and NBS-LRR genes was induced by saline or heat stresses or by infection with *Erwinia carotovora*, respectively. The transcriptional activation was also higher in *A. angustifolia* Haw. 'Chelem Ki' ($2n=6x=180$) and *A. fourcroydes* 'Sac Ki' ($2n=5x=150$) than in their diploid and triploid counterparts, which suggests higher adaptation to stress. Finally, the diploid accession *A. tequilana* Weber 'Azul' showed a differentiated genetic profile relative to other *Agave* accessions. The differences include similar or higher genetic representativeness and transcript accumulation of LEA and NBS-LRR genes than in polyploid ($2n=5x=150$ and $2n=6x=180$) *Agave* accessions, thus suggesting a differentiated selection pressure for overcoming the lower ploidy level of the diploid *A. tequilana* Weber 'Azul'.

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1. Introduction

Agave L. is a genus including 166 species mainly distributed in Mexico, is characterized by its wide ploidy range ($2n=2x=60$ to $2n=8x=240$) (Baneerje and Sharma, 1987; Castorena-Sánchez et al., 1991; Robert et al., 2008), the presence of allopolyploid genomes (McKain et al., 2012), and the xerophytic adaptations (Nobel, 1976; Nobel and Hartsock, 1978, 1979; Pimienta-Barrios et al., 2005a,b) that allowed it to inhabit a wide range of environments. Polyploidy is commonly recognized as having played a critical role in plant evolution and speciation (Wendel, 2000). Recent studies suggested that polyploidy species, and specifically allopolyploid species, gain advantages from the control of circadian-mediated physiological and metabolic pathways leading to vigorous growth and increased biomass (Chen, 2010; Ni et al., 2009). Allopolyploidy can be associated with an increased number of gene copies and, therefore, is involved with the generation of redundant genes (Lee and Chen, 2001). These genes, mainly generated by duplication, are involved in epigenetic regulation (Buggs et al., 2012; Lee and Chen, 2001), become specialized to perform a complementary function (Proulx, 2012; Roque et al., 2012; Ward and Durrett, 2004), or may eventually be lost (Buggs et al., 2012;

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Pikaard, 2001; Soltis and Soltis, 1999; Wendel, 2000). The functional partitioning and differences in expression patterns of these redundant genes are associated to the physiological and phenotypic changes (Allario et al., 2011; Aversano et al., 2013; Chen 2007; Stupar et al., 2007) needed for supporting these advantages in allopolyploid species. Some plants including polyploid species (i.e. *Robinia pseudoacacia* L., *Lonicera japonica* Thunb., *Citrus sinensis* Osbeck., *Atriplex canescens* (Pursh) Nutt and *Triticum* spp.) are more tolerant to biotic or abiotic stresses in comparison to their diploid counterparts, as described by Hao et al. (2013), Li et al. (2009), Meng et al. (2009), Shafieizargar et al. (2013), Wang et al. (2013), and Yang et al. (2014). However, this comparison of stress tolerance between polyploid and diploid species has not yet been conducted in the genus *Agave*. Recent studies in *Agave* plants have shown that the number of ribosomal copies increases with ploidy level (Robert et al., 2008), and that this increase could also generate redundant and non-redundant ribosomal copies displaying different expression profiles (Tamayo-Ordoñez et al., 2015). Shakeel et al. (2013), in a research focused in exploring the transcriptomic and proteomic changes in response to heat stress of *Agave americana* L., showed a differential expression of genes related to stroma as a response to abiotic and biotic stresses that was uncorrelated with the plant's proteomic profile. The transcriptomic changes of these genes could be related to the high number of copies, to organization, and to regulation. In fact, all these genetic and regulatory changes in stress-related genes provide species of *Agave* a better response against environmental stresses.

LEA and NBS-LRR genes are involved in physiological responses to stresses such as pathogenic infection, salinity, and temperature in plant species such as, for example, *Brassica napus* L., *Jatropha curcas* L., *Glycine max* (L.) Merr., *Arabidopsis* Heynh in Holl & Heynh, *Malus* × *domestica* Borkh and *Saccharum officinarum* L. (Arya et al., 2014; Chini et al., 2004; Dalal et al., 2009; Liang et al., 2013; Savitri et al., 2013; Selvaraj et al., 2013). Additionally, Martínez-Hernández et al. (2010) observed in species of *Agave* the transcription of LEA in field conditions and the presence of NBS-LRR protein under heat stress conditions (Shakeel et al., 2013), which suggests an important role of these genes during periods of stressful abiotic and biotic conditions, respectively.

To date, it is still unknown if the polyploid species of *Agave* can respond better to environmental stress in comparison to lower ploidy level counterparts and what are the phenotypic and molecular factors related to responses of *Agave* to environmental stresses. Aiming at answering these questions, in this study we characterized the genetic and morphological response to stress of *Agave* accessions with different ploidy levels ($2n = 2x = 60$ to $2n = 6x = 180$). This characterization included leaf morphology, and the representativeness and expression profile of the NBS-LRR gene after *Erwinia carotovorum* infection, and of the LEA gene under saline or heat stresses. Our results indicated that each analyzed *Agave* accession has phenotypic differences, which together with the genetic background and transcript accumulation of genes related to stress could help these species to better respond to environmental stress, and are showing the complex mechanisms present in polyploid *Agave*.

2. Material and methods

2.1. Plant materials

Three *Agave* species (*A. tequilana* Weber 1902, *A. fourcroydes* Lem. 1864, and *A. angustifolia* Haw. 1915), two of which included variants with different ploidy levels, were selected for this study. The selected accessions were: *A. tequilana* Weber 'Azul' ($2n = 2x = 60$), *A. angustifolia* Haw. 'Marginata' ($2n = 2x = 60$), *A. angustifolia* Haw. 'Chelem ki' ($2n = 6x = 180$), *A. fourcroydes* Lem. 'Kitam ki' ($2n = 3x = 90$), and *A. fourcroydes* Lem. 'Sac ki' ($2n = 5x = 150$). Five plants were included of each *Agave* accession, with a total of 25 plants analyzed. Robert et al. (2008) and Tamayo-Ordoñez et al. (2015) previously analyzed ploidy and its relation with ribosomal gene loci, respectively. All these accessions, with the exception of *A. tequilana* Weber 'Azul' ($2n = 2x = 60$), were directly collected from the field and adapted to greenhouse conditions until their analysis. *A. tequilana* Weber 'Azul' ($2n = 2x = 60$) was initially collected in a commercial plantation and successively propagated for more than 5 years by *in vitro* culture. In *A. tequilana* Weber 'Azul' ($2n = 2x = 60$) explants were adapted to greenhouse conditions until their present analysis. All *Agave* accessions are maintained in the installations of the Research Center of Yucatan (CICY), México.

2.2. Characterization of stress adaptations

The macroscopic and microscopic morphological measurements analyzed with the aim of knowing the specific basal adaptations exhibited by each *Agave* accessions had been previously associated to physiological adaptations to stress responses in plants (Doheny-Adams et al., 2012; Frank et al., 2011; Gutschick, 1999; Vandenhout et al., 1995; War et al., 2012) and included: leaf length, leaf base width, leaf middle with, leaf tip width, total leaf area, terminal spine length, marginal spine length, total number of spines, nuclei number, nuclei size, stomatal area, stomata guard cell area, suprastomatic cavity area, abaxial and adaxial stomata density, and total stomata density. Each calculated parameter by accession includes data obtained from 3 leaves of 5 plants of each *Agave* accession.

Leaf macroscopic characters were independently calculated by two strategies: first, by an average of three leaf sections (inner, middle and top relative to the stem); and second, by image digitalization (3 leaves of 5 plants of each accession) covering all the leaf by using the Image tool software ver. 3.0 (Wilcox et al., 2002). Stomatal size and density were calculated from the central regions of leaves both from the adaxial and the abaxial epidermis using SEM analysis. This analysis was carried out as follows: 1 cm^3 of sample was fixed in 2.5% glutaraldehyde for 48 h, rinsed several times in buffer solution (0.2 M sodium phosphate, pH 7.1), and dehydrated in serial ethanol concentrations (v/v) of 30%, 50%, 70%, 85% and 96%. All these dehydrated rinses were sequentially followed by a rinse in absolute ethanol for 60 min. The critical drying point was performed at 1072 psi/31 °C (Samdri®-795 tousimis). The samples were then mounted on metallic stubs with carbon conductive adhesive tape (Electron Microscopy Science) and sputter coated with a 150 Å gold layer (Denton Vaccum Desk II). Stomatal size and density were calculated for the abaxial and adaxial epidermis at a magnification of 100× (0.1213 mm^2). Length of the guard cells was also measured in five stomata per field. Counts and measurements were done in ten fields of each leaf in each accession. Sample analysis and image recording were made using a Scanning Electron Microscope (Jeol, JSM-6360LV). Size and number of nuclei determinations were made from a nuclei solution obtained from 1 cm^3 of leaves from the sections closest to the basal rosette according to the procedures given by Tamayo-Ordóñez et al. (2012). Nuclei integrity and size were confirmed by optical microscopy (magnification of 100×) and their quantification was carried out using a hemocytometer. Finally, morphological values were correlated with ploidy level in each *Agave* accession by using a polynomial ($k = 2$) regression to identify those parameters responding to ploidy level.

2.3. Phylogenetic analysis of LEA and NBS-LRR genes

Genomic DNA was isolated from young leaves by the silica method (Echaverria-Machado et al., 2005). LEA and NBS-LRR ampli-

fication were conducted using the primers previously reported by Tamayo-Ordóñez et al. (2012). PCR reactions were carried out in a volume of 50 μL containing 25 ng of genomic DNA, 130 μM dNTPs, 15 μM of each primer, 2.5 units of *Taq* polymerase, and 1X PCR reaction buffer (Life Technologies, Rockville, MD, U.S.A.) with 1.5 mM $MgCl_2$. PCR conditions included 1 cycle of 3 min at 94 °C for initial denaturation, followed by 35 cycles of 1 min at 94 °C, 1.5 min at 60 °C, 1 min at 72 °C, and finally 7 min at 72 °C. PCR products were separated by electrophoresis in 1.2% agarose gels. Desired bands of 350 bp (LEA) and 400 bp (NBS-LRR) were afterwards gel purified using the Wizard® SV Gel and PCR clean up System (Promega, U.S.A.). Purified DNA was cloned into pGEM-Teasy vector according to manufacturer instructions (Promega, Madison, WI, U.S.A.). Five events were performed by cloning from each accession (from the DNA of 5 individuals) and for each cloning event, 10 clones were selected and sequenced, giving a total of 30 sequences per gene and per accession. The external company MACROGEN (Seoul, Korea) sequenced the selected LEA and NBS-LRR clones. With the goal of minimizing sequencing errors we decided to sequence each clone using forward and reverse M13 universal primers. We verified that the forward and reverse sequences of each fragment were identical. Only sequences showing no errors in nucleotides were included in the analyses.

The nucleotide sequences were aligned (BLASTX) and compared with those in the GenBank database. DNAMAN version 4.0 was used to translate these sequences and to identify the open reading frame. Nucleotide sequences obtained in this study were deposited in the GenBank database. Accession numbers corresponding to sequences of NBS-LRR gene are KU295166, KU95167, KU29568, KU295169 and KU295170. Sequences of LEA gene are KU315010, KU315011, KU315012, KU315013 and KU315014. Predicted amino acid sequences relative to the LEA and NBS-LRR nucleotide sequences were used in combination with related sequences retrieved from GenBank to build a phylogenetic aligning. Conserved aligned regions (>90%) were selected in all sequences analyzed in order to build dendrograms by the MEGA 4.1 program (Tamura et al., 2007) using the neighbor-joining method (Saitou and Nei, 1987). The reliability of the clusters was evaluated by bootstrapping with 1000 replicates.

2.4. Abiotic stress conditions related to LEA genes

LEA genes are involved with physiological response to abiotic stresses such as salinity or temperature in many species (i.e. *Tamarix hispida* L., *Hordeum vulgare* L., *Triticum* spp. L., *Arabidopsis thaliana* Heynh in Holl & Heynh and *Oriza sativa* L.) (Duan and Cai, 2012; Gao et al., 2014; Liang et al., 2013; Kosová et al., 2014). Stressful conditions of salinity and temperature were induced in all studied *Agave* accessions. Physiological responses were analyzed in five individuals of each accession, plants grown from bulbils produced asexually from the previously mentioned original mother plants. All these *Agave* plants were grown in inert material (agrolite-sand, 50:50) supplemented with periodical irrigations of 500 mL of Hoagland solution (Hoagland and Arnon, 1957) each 4 days during one year prior to conducting the physiological studies.

Salinity stress was induced by irrigating these 1-year-old plants with 500 mL of Hoagland solution supplemented with 250 mM of NaCl during two periods (15 and 30 days). At the end of each stressful period, leaf tissue (external leaves) was collected in liquid nitrogen and stored at −80 °C until further analysis. All experiments were conducted by triplicate for each individual 5 plants and for each accession.

In parallel, temperature stress was induced in these 1-year-old plants by placing them in an incubation chamber at 40 °C with similar light and irrigation conditions as suggested by Shakeel et al. (2013). Two periods of temperature stress were applied at 15 and 30 days. After each stressful period, leaf tissue (external leaves) was collected in liquid nitrogen and stored at −80 °C for further analysis. All experiments were conducted by triplicate for each of the five individual plants and for each accession, and unstressed *Agave* plants adapted and grown in the greenhouse were used as negative controls for salinity and temperature stress experiments.

2.5. Biotic stress conditions related to NBS-LRR genes

NBS-LRR genes are involved with the physiological response to pathogenic infection (biotic stress) in many species (Arya et al., 2014; Chini et al., 2004; Kang et al., 2012; Wan et al., 2012). We evaluated the response of gene NBS-LRR after *Agave* plants were infected with *E. carotovorum* (Smith) Yabuuch because this bacteria causes bud rot and death of *Agave* plants after the larvae of the Agave snout weevil – *Scyphophorus acupunctatus* Gyllenhal (Coleoptera: Curculionidae) – bores the leafs (Solís-Aguilar et al., 2001).

Pathogenic infection was induced in all *Agave* accessions of this study. For that, meristematic tissue explants (~1 cm^3) were obtained from 1-year-old plants and placed in Murashige and Skoog broth culture (Murashige and Skoog, 1962) during 2 months at constant light and 22 °C to allow their adaptation to these conditions. Afterwards, these explants were infected with 3×10^6 UFC of an *E. carotovorum* isolate with infective potential over different plant species (Norman-Setterblad et al., 2000). After infection, 100 mg of these meristematic explants (infected and non-infected) were collected in liquid nitrogen at 0.5, 5 and 12 h and stored at −80 °C for further analysis. All experiments were conducted in every studied *Agave* accession, including five individuals per accession, also including the corresponding specific uninfected counterpart, and by triplicate.

2.6. Determination of copy number per genome of genes LEA and NBS-LRR in Agave

2.6.1. Building of standard calibration curves

Copy number per genome of the LEA and NBS-LRR genes was determined by the method of Lee et al. (2008) and Lu et al. (2012) with modifications. In order to avoid overestimation of copy number of the analyzed genes in complex polyploid genomes we built two types of calibration curves: one specific for each of the studied genes (standard gene curve), and a second one specific for each analyzed accession (standard accession curve) (Fig. S1). The standard gene curve was built from the clones of LEA and NBS-LRR genes (named LEA-pGEM and NBS-LRR-pGEM) that had been obtained in the previous phylogenetic analysis in six serial dilution series for each plasmid (10, 1, 0.1, 0.01, 0.001 and 0.0001 ng/μL). By means of sequencing, it was verified that each clone contained only one copy of each gene. Each concentration of plasmids used for building the standard curves was converted to copy number according to the formula proposed by Whelan et al. (2003) and Lu et al. (2012): DNA (copy) = 6.02×10^{23} (copies mol^{-1}) × DNA amount (g)/DNA length (bp) × 660 (g mol^{-1} bp^{-1}). In both genes, the number of PCR cycles for the fluorescence signal to reach a value above that of the background fluorescence (C_T cycle threshold value) was determined according to the fluorescence threshold intensity by using the StepOne Software v2.0 (Applied Biosystems). The C_t values were plotted against the logarithm of their initial template copy concentrations. Each standard curve was generated by linear regression of the plotted points. Following Yuan et al. (2007), a regression analysis was run for each standard curve. The Regression Procedure in SAS was first executed to model *Ct* value against log-transformed input DNA concentration. For data quality control purpose, the test statement establishing the *Ct* equivalence to −1 helps to estimate

if the slope of the simple linear regression is −1 and indicates an amplification efficiency of 100%. Additionally, PCR amplification efficiency (*E*) was calculated from the slope of each curve by the formula $E = 10^{-1/slope} - 1$.

The standard accession curve was made using a nuclei solution obtained from one-year-old external leaves, because it was previously reported that the 4C DNA content vary widely among different *Agave* species (Greilhuber et al., 2005; Moreno-Salazar et al., 2007; Palomino et al., 2003; Robert et al., 2008; Tamayo-Ordoñez et al., 2015), so that the use of the same concentration of DNA (ng/μL) without standardizing the gDNA content in each polyploid species could affect the representatives of copy number in the genome. The extraction of isolated nuclei was conducted following procedures given by Zhang et al. (1995). Nuclei integrity was confirmed by optical microscopy and nuclei quantified in a hemocytometer. In all specimens, two curves were built by amplification of each studied gene across several nuclei concentrations (10; 100; 1000; 10,000; and 100,000 nuclei mL^{-1}). Each nuclei concentration was used for gDNA isolation according to Echaverria-Machado et al. (2005). The total gDNA extracted for each nuclei concentration was resuspended in 20 uL de nuclease-free H_20. Quantity, quality and purity of the DNA were evaluated using NANODROP equipment (NANODROP, Promega, USA). The master mix reaction consisted of 1uL of gDNA from each nuclei dilution in 20 μL of PCR reaction mix containing 10 μL of PCR Master Mix (2X) with SYBR Green (Applied Biosystems) and 6 μM of forward and reverse primers.

Primers and PCR amplification conditions were the same as previously described for phylogenetic analysis of the LEA and NBS-LRR genes. With the aim of discarding fluorescence originated from nonspecific PCR products and primer concatenation, a melt-curve analysis was performed. In order to eliminate DNA contamination negative controls for each gene were always included in the experiments. The SYBR green fluorescent signal was standardized to a passive reference dye (ROX) included in the SYBR Green PCR Master Mix (Applied Biosystems P/N 4309158).

Ct values for each point in the standard accession curve was extrapolated to the standard gene curve in order to estimate the number of gene copies present in each sample, and multiplied by 20 given that total DNA solutions were previously resuspended in a final volume of 20 μL. Finally the nuclei concentrations (10; 100; 1000; 10,000; and 100,000 nuclei mL^{-1}) were inferred to gDNA according to the genome size of each species. Therefore, for each accession and gene analyzed two types of plots were made: gDNA concentration vs. *Ct*, and gDNA concentration vs. gene copy number. Amplification efficiency values were calculated as formerly described.

2.6.2. Estimation of NBS-LRR and LEA gene copy numbers in Agave accessions

We quantified the copy number per genome (representativeness) of genes NBS-LRR and LEA by the method of absolute quantification (Chini et al., 2007; Lee et al., 2008; Lu et al., 2012). Absolute quantification determines the exact copy concentration of the target gene by relating the C_t value to a standard curve (Fig. S1A).

Based on data from the calibration curve for nuclei the amount of nuclei was previously standardized in all specimens to 30,000 nuclei mL^{-1}, because that nuclei concentration resulted in the best values of *Ct* for estimating the absolute copy number, always at 100% amplification efficiency. Extraction, integrity, and gDNA isolation was made as previously described. Afterwards, 50 ng of DNA was used as template in 20 μL of PCR reaction mix containing 10 μL of PCR Master Mix (2X) with Syber Green (Applied Biosystems) and 6 μM of forward and reverse primers. Primers and PCR amplification conditions were the same as previously.

Finally the gene and accession standard curves were used for interpreting data. The *Ct* values for each accession were interpolated in the pDNA curve (standard gene curve). Later on, in order to know how much gDNA corresponded to that copy number, the standard accession curve was used for interpolating the estimated number of copies in the gDNA concentration vs. copy number plot for each studied accession and gene (Fig. S1C).

2.7. Determination of the expressed copy number and relative expression of genes NBS-LRR and LEA in Agave

RNA isolation and cDNA synthesis were conducted according to Tamayo-Ordoñez et al. (2015). High quality RNA was obtained from a constant number of nuclei (30,000 nuclei mL^{-1}) regardless of the ploidy levels of the *Agave* accessions assayed. RNA isolation was conducted using the TRIZOL method (Invitrogen, USA) by following the manufacturer's protocol. RNA integrity and quantification were verified in 1.3% agarose gels after electrophoresis and by spectrometry using the A_{260}/A_{280} and A_{260}/A_{230} ratios in NANODROP (NANODROP-1000, ThermoScientific, USA). 1 μg total RNA was digested with 1U RQ1 RNase-Free DNase I (PROMEGA) and DNase 1X reaction buffer in a final volume of 10 μL. The samples were incubated at 37 °C for 30 min. Subsequently the DNase I was inactivated by adding 1 μL of RQ1 DNase Stop Solution and incubating at 65 °C for 10 min. The absence of DNA contamination was verified by agarose gel electrophoresis (1.3%) and negative PCR, using as a template 50 ng of RNA treated with DNase I. In this PCR a sample of genomic DNA was included as a positive control. The primers and PCR amplification conditions were the same as previously described for phylogenetic analysis of the LEA and NBS-LRR genes.

The cDNA was synthesized using the GoScript™ Reverse Transcription System (PROMEGA, USA) following manufacturer's instructions and the previous report of Tamayo-Ordoñez et al. (2015). A positive control (1.2 Kb Kanamycin Positive Control RNA) provided in the kit was used to estimate the yield of synthesis of cDNA. The concentrations of cDNA were verified by NANODROP measurements. The cDNAs obtained were stored at −60 °C until its use.

Amplification of the NBS-LRR and LEA genes was carried out as before. PCR amplification conditions were the same as described above for the determination of gene copy number. The melt curve analysis and negative controls for the reference and target genes were always included in the experiments in order to eliminate DNA contamination. Finally two analyses were carried out, the transcripts copy numbers (absolute RT-qPCR) were obtained extrapolating C_t values obtained from cDNA in all *Agave* accessions with the values of standard curves. The standard curves applied were the same as those used in the quantification of absolute of copy number in the genome as described above. We analyzed data with the standard gene and accession curves, first interpolating the *Ct* value to estimate a copy number in the gene standard curve, and afterwards, interpolating the estimated copy number in the standard accession curve for estimating the number of expressed gene copies relative to the proportion of genome analyzed. The final values were adjusted considering the genome sizes (ploidy level) of each accession (Fig. S1B y S1C). Finally, the absolute number of expressed copies – under the analyzed conditions and taking into account the copy number per genome (representativeness) of genes LEA and NBS-LRR for each species of *Agave* – is represented by the number of transcribed copies of these genes. In order to overcome the differences in ploidy and gene representativeness among the studied *Agave* accessions, the numbers of transcribed copies were plotted against the total copy number present in the gDNA.

The relative expression of each gene was determined by the $\Delta\Delta$Cq method between the target (NBS-LRR and LEA) and refer-

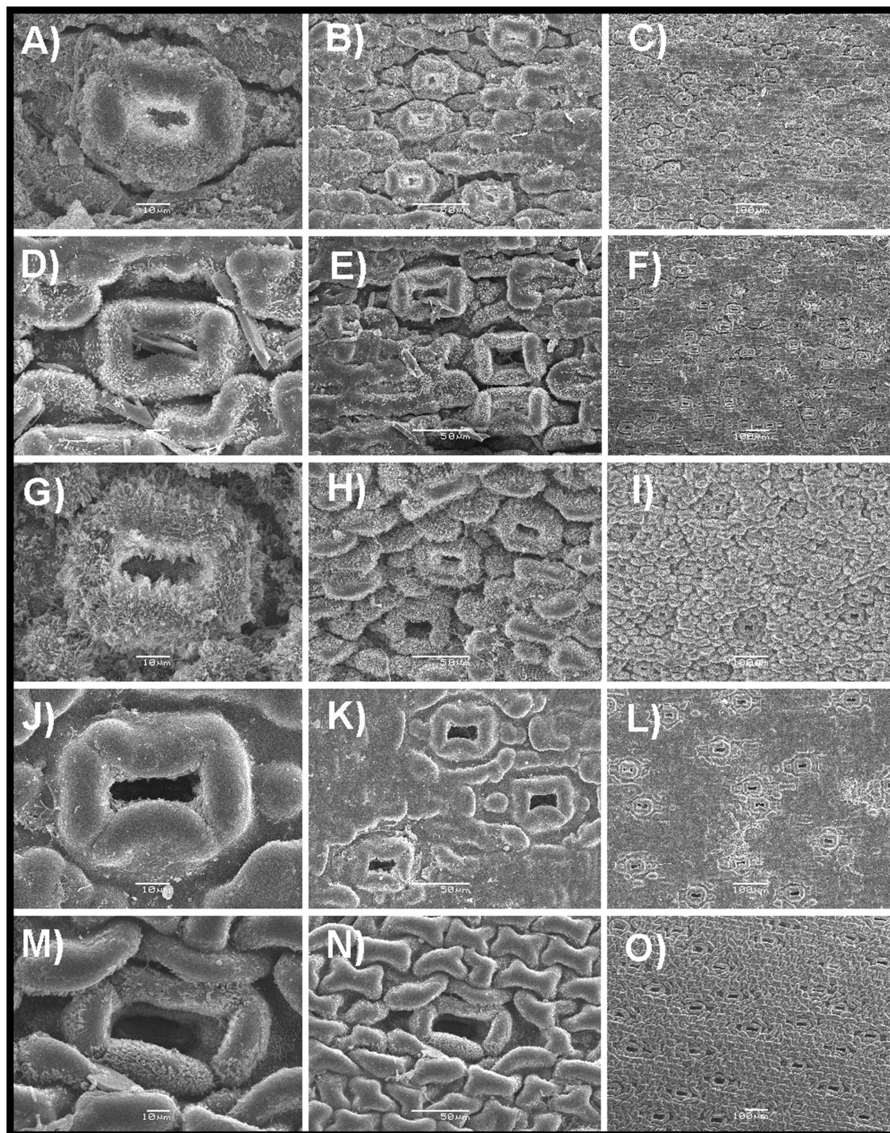

Fig. 1. Scanning electron microscopy of stomata in the *Agave* L. accessions of study. **A–C** are *A. tequilana* Weber 'Azul' ($2n = 2x = 60$); **D–F** are *A. angustifolia* Haw. 'Marginata' ($2n = 2x = 60$); **G–I** are *A. fourcroydes* Lem. 'Kitam ki' ($2n = 3x = 90$); **J–L** are *A. fourcroydes* Lem. 'Sac ki' ($2n = 5x = 150$); **M–O** are *A. angustifolia* Haw. 'Chelem ki' ($2n = 6x = 180$). Right, middle, and left bars measure 10 μM, 50 μM and 100 μM, respectively.

ence (18S rDNA) genes (Maroufi et al., 2010; Nicot et al., 2005; Pfaffl, 2001). The 18S rDNA gene was selected as a reference because in *Agave* plants it has shown conservation of sequence, and because in previous RT-qPCR analysis it has proven to be a reliable reference as it has ubiquitous expression across different field conditions (Tamayo-Ordoñez et al., 2015). The transcript abundance ratio of target gene to reference gene was determined by the following equation: Relative Expression = $(E_{ref})^{Ctref}/(E_{target})^{Ctarget}$, where E_{ref} and E_{target} are the efficiencies of the primers for the reference and target genes, respectively, and Ct_{ref} and Ct_{target} are the mean C_t value of the reference and target genes, respectively (Pfaffl, 2001). For the analysis of gene expression of NBS-LRR and LEA, relative expression of both genes was calculated for each condition and the values of relative expression in the corresponding controls were subtracted.

2.8. Detection of LEA and NBS-LRR genes

The LEA and NBS-LRR genes were detected in cell nuclei isolated from meristematic tissues by fluorescence in situ hybridization (FISH) analysis. Nucleic cells were isolated as described above. These plant tissues have previously been reported as reliable for application of FISH in plants (Tirichine et al., 2009). LEA and NBS-LRR probes were selected from a previously obtained BIBAC library (Tamayo-Ordóñez et al., 2012). Probe selection was conducted by qPCR on plasmidic DNA (50 ng/μL) by using the previously described primer sets and PCR conditions. Clones −85 and −78 of the LEA and NBS-LRR genes were selected by their highest copy number according to the qPCR analysis (data not shown). LEA and NBS-LRR clone labeling was carried out with digoxigenin-11-dUTP by a nick translation strategy (Roche Applied Science). Fixation, hybridization and immunodetection were done according to Robert et al. (2008). Preparations were mounted and counterstained with Vectashield (Vector Laboratories) containing 2.5 μg μL^{-1} of DAPI (40,6-diamidino-2-phenylindole; Serva) or FITC (fluorescein isothiocyanate, Sigma, Aldrich) for nuclei or gene detection, respectively. Microscopy analysis was conducted in an epifluorescence microscope (Carl Zeiss Axioplan Jena, Germany). Image analyses were carried out by Carl Zeiss AxioVision software. The obtained images

were pseudocolored, merged, and processed in the Adobe Photoshop program (Adobe Systems, Mountain View, CA).

2.8.1. Statistical analysis

Collected data were subjected to analysis of variance (ANOVA) using the SAS statistical software package ver. 9.0 (2000) and Origin 9.1 Software (Data Analysis and Graphing Software). Statistical *F*-tests were evaluated at <0.05 and the means were compared by Tukey tests (P > 0.05).

3. Results and discussion

3.1. Morphological differences and adaptations in studied accessions of Agave

Macroscopic and microscopic determinations were obtained from three leaves of 5 plants for each accession. Three types of comparisons were made for each characteristic: (1) between ploidy level irrespective of species, (2) between ploidy levels within the same species, and (3) between species having the same ploidy level. This analysis indicated if differences between analyzed characteristics were more related to ploidy level within each species, to ploidy level irrespective of species, or to species being analyzed. *Agave tequilana* Weber 'Azul' ($2n = 2x = 60$) was used as a diploid control when comparing results with *A. angustifolia* Haw. 'Marginata' ($2n = 2x = 60$), and the differences found between these diploid species were related to the particular adaptive characteristics of the species and not to ploidy level.

Macroscopic determinations (length or width) on the leaves showed more differences (Tukey test; P > 0.05) among the *Agave* accessions than among ploidy levels (Fig. S2; Table 1). Only two characters showed no change, leaf length and marginal spine length. Leaf length had an average of 71.12 cm among all *Agave* accessions of study (Fig. S2), which suggests leaf length is unrelated to ploidy level and is not an adaptive morphological character in the studied species. However, spine values require further analysis since the length of the terminal spines is up to 2.2-fold longer in *A. angustifolia* Haw. 'Chelem ki' ($2n = 6x = 180$) than in *A. tequilana* Weber 'Azul' ($2n = 2x = 30$) and *A. angustifolia* Haw. 'Marginata' ($2n = 2x = 60$) (Fig. S2A), an interspecific difference that closely ($R^2 = 0.9584$) correlates with ploidy level. Similarly, total spine number was larger in the polyploid species *A. fourcroydes* Lem. 'Kitam ki' ($2n = 3x = 90$), *A. fourcroydes* Lem. 'Sac ki' ($2n = 5x = 150$), and *A. angustifolia* Haw. 'Chelem ki' ($2n = 6x = 180$) than in diploid *A. angustifolia* Haw. 'Marginata' ($2n = 2x = 60$). However, *A. tequilana* Weber 'Azul' ($2n = 2x = 30$) had the highest total spine number, which points to spine number having a closer relation to species than to ploidy level, but within species that number also depends on ploidy level, as shown in accessions of *A. angustifolia* Haw.

Micromorphological comparisons between abaxial and adaxial leaf-epidermis confirmed anatomical differences among all *Agave* accessions (Table 1; Fig. 1). In all *Agave* accessions styloid crystals surrounding the suprastomatic cavity (Fig. 1D–F) were apparent. In addition, in all *Agave* accessions we observed groups of 3–5 stomata arranged in short rows (Fig. 1C, F, I and J). Only three microscopic characters (Nuclei number, Nuclei length, and Suprastomatic cavity area) exhibited some sort of relation with ploidy level (Fig. S4). Nuclei length directly increases ($R^2 = 9.394$) according to ploidy level, whereas the density of nuclei (number per cm^2) decreases ($R^2 = 0.6082$) with ploidy level. The suprastomatic cavity area also varied according to ploidy level ($R^2 = 0.8899$), being 3.6 times larger in *A. angustifolia* Haw. 'Chelem ki' ($2n = 6x = 180$) than in *A. angustifolia* Haw. 'Marginata' ($2n = 2x = 60$) (Fig. S3). However, the stomatal density was highest (average 80.5 mm^{-2}) in *A. tequilana* Weber 'Azul' ($2n = 2x = 30$) and *A. angustifolia* Haw. 'Marginata' ($2n = 2x = 60$) (Table 1). Therefore, two micro-phenotypic (micromorphological) coefficients were proposed: the nuclei coefficient relating nuclei number with nuclei size, and the stomatal coefficient relating the area of the suprastomatic cavity with stomatal density. Both micro-phenotypic coefficients displayed high correlations ($R^2 > 0.9$) with ploidy level (Fig. 2).

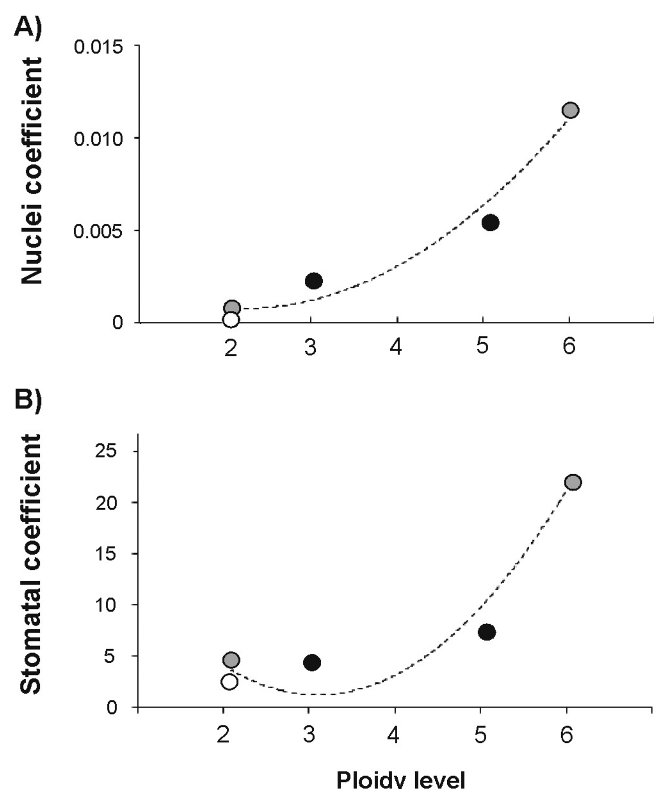

Fig. 2. Phenotypic coefficients showing relation with ploidy level in the studied accessions of *Agave* L. (**A**) Nuclei coefficient [Nuclei number/Nuclei size (μM)], and (**B**) Stomata coefficient [Suprastomatic cavity area (μM^2)/Stomata density (stomata number/mm^2)]. Symbols: (○) *A. tequilana* Weber 'Azul' ($2n = 2x = 60$), (●, grey) *A. angustifolia* Haw., and (●) *A. fourcroydes* Lem.

Morphological characterization of the leaves of the studied *Agave* accessions shows a probable relation between spine, nuclei and stomatal morphological values and ploidy level. Nuclei size and number, and stomatal length and density have been described in polyploid varieties of important crop plants (Balao et al., 2011; Byrne et al., 1981; Jellings and Leech, 1984). In our study, nuclear and stomatal densities varied according to the ploidy level. This nuclear phenomenon known as *gigas* has been described in other polyploids plants (Lutz, 1907; Tupper and Bartlett, 1916), and has been related with stress adaptation. For instance, in *Lycopersicon esculentum* Mill., high temperatures promoted an increase in cell size with a slight reduction in cell number (Bertin, 2005). High temperature could also play a role in the progression rate of nuclei from a lower to a higher C-value, and contribute to the increase in ploidy level. In *Rosa* sp., temperatures above 36 °C cause the increase of ploidy level in gametes, supporting the hypothesis of polyploidization events occurring in adverse conditions (Pécrix et al., 2011), and also suggesting that the *gigas* phenomenon in *Agave* plants might be the result of adaptation to heat stress. In the *Agave* accessions we studied stomatal densities were also related to ploidy level. Some plants such as *Betula papyrifera* Marsh. and *A. thaliana* Heynh in Holl & Heynh have been show to have fewer stomata per unit area and smaller stomatal indices than their diploid counterparts (Li et al., 1996, 2012). Stomatal indices according to ploidy numbers have also been related to adaptation to stress (Balao et al., 2011). Accord-

Table 1
Morphological determinations in the studied accessions of *Agave* L.

Characteristics[c]	*A. tequilana* Weber 'Azul' (2n = 2x = 60)[a,b]	*A. angustifolia* Haw. 'Marginata' (2n = 2x = 60)[a,b]	*A. fourcroydes* Lem. 'Kitam ki' (2n = 3x = 90)[a,b]	*A. fourcroydes* Lem. 'Sac ki' (2n = 5x = 150)[a,b]	*A. angustifolia* Haw. 'Chelem ki' (2n = 6x = 180)[a,b]
Macroscopic					
Leaf length (cm)	72.83 ± 6.84[a]	69.33 ± 5.98[a]	74.37 ± 0.74[a]	73.97 ± 13.60[a]	65.10 ± 16.41[a]
Leaf base width (cm)	2.77 ± 0.38[a]	4.83 ± 0.50[b]	2.53 ± 0.35[a]	2.73 ± 0.38[a]	3.03 ± 0.06[a]
Leaf middle width (cm)	4.30 ± 0.26[a]	7.60 ± 0.61[b]	4.00 ± 0.66[a]	4.40 ± 0.53[a]	5.90 ± 0.66[b]
Leaf tip width (cm)	2.53 ± 0.25[b]	1.03 ± 0.32[a]	1.73 ± 0.32[b]	2.10 ± 0.17[b]	1.87 ± 0.40[b]
Total leaf area (cm^2)	257.80 ± 4.20[b]	261.00 ± 3.46[b]	288.00 ± 1.61[d]	233.00 ± 3.82[a]	273.00 ± 4.26[c]
Terminal spine length (cm)	1.03 ± 0.23[a]	1.33 ± 0.20[a]	1.67 ± 0.25[b]	2.20 ± 0.30[b]	2.20 ± 0.10[b]
Marginal spine length (cm)	0.19 ± 0.06[a]	0.16 ± 0.04[a]	0.13 ± 0.03[a]	0.20 ± 0.05[a]	0.16 ± 0.05[a]
Total number of spines	148.00 ± 7.00[c]	50.00 ± 7.00[a]	94.00 ± 9.00[b]	78.00 ± 8.00[b]	74.00 ± 3.00[b]
Microscopic					
Number of nuclei/cm^2	11,236.00 ± 64.00[e]	31,687.00 ± 65.00[d]	5,155.00 ± 69.00[c]	3,364.00 ± 80.00[b]	1,936.00 ± 48.00[a]
Length of nuclei (μM)	9.40 ± 0.32[b]	5.60 ± 0.56[a]	12.53 ± 75.00[c]	17.20 ± 0.64[d]	22.50 ± 0.20[e]
Stomata area (μM^2)	1,043.00 ± 64.00[b]	1,637.00 ± 70.00[c]	997.00 ± 82.00[a]	1,462.00 ± 135.00[c]	2,774.00 ± 39.00[e]
Guard cells area (μM^2)	846.00 ± 36.00[a]	1,278.00 ± 68.00[b]	794.00 ± 71.00[a]	1,195.00 ± 104.00[b]	793.00 ± 37.00[a]
Suprastomatic cavity area (μM^2)	196.00 ± 30.00[a]	358.00 ± 1.25[c]	203.00 ± 14.00[a]	266.00 ± 31.00[b]	1,315.00 ± 6.00[d]
Abaxial stomata density (number mm^{-2})	42.00 ± 1.76[c]	51.50 ± 1.50[d]	23.00 ± 2.40[a]	20.00 ± 0.66[a]	26.00 ± 3.00[b]
Adaxial stomata density (number mm^{-2})	40.00 ± 0.60[d]	28.00 ± 1.50[b]	26.00 ± 1.15[b]	18.00 ± 0.66[a]	32.00 ± 2.50[c]
Total stomata density (number mm^{-2})[c]	82.00 ± 2.36[c]	79.00 ± 3.00[c]	49.00 ± 3.55[b]	38.00 ± 1.32[a]	58.00 ± 5.50[b]

[a] Lowercase letters (a, b, c, or d) indicate significantly different values (Student's *t*-test; $p < 0.05$).
[b] Values are averages of triplicates obtained from 3 leaves of five plants of each accession ± standard error.
[c] Calculated by adding the values of the abaxial and adaxial stomata densities.

ingly, previous studies described a direct relation between water deficit, stomatal dimensions, and ploidy level affecting the plant's uptake of water (Li et al., 1996, 2012). Other study conducted in *Lycopersicum esculentum* Mill. by Salas et al. (2001) showed that solar radiation has also been involved in the reduction of stomatal density, thus supporting the above-mentioned polyploidization hypothesis. Adaptations preventing physiological damages due to draught reported for *Agave* plants include nocturnal assimilation of CO_2, thick cuticles, low stomata density, and succulent leaves. The latter two adaptations enable water stored in the leaf parenchyma to continuously move to the chlorenchyma during dry periods (Pimienta-Barrios et al., 2005a,b, 2006), thus conferring the plants the capacity for withstanding up to seven years of draught (Stewart, 2015).

At the macroscopic level, the length of the terminal spine also exhibits a direct relation with ploidy number. Spines have been associated to defense against herbivores and protection from heat stress (Gibson and Nobel, 1986; Goldstein and Nobel, 1994; Mosco, 2009; Young et al., 2003). Our study also found styloid crystals surrounding the suprastomatic cavity in all *Agave* accessions, which could indicate a possible adaptation against plant predators, since these calcium oxalate crystals can be used as a defense mechanism (Hudgins et al., 2003; Nakata 2012; Korth et al., 2006). Polyploidy is a very common phenomenon in species of angiosperms and is considered to be involved in evolution and speciation (Madlung, 2013; Moghe and Shiu, 2014; Wendel, 2000). However, it is here important to mention that the diploid ($2n = 2x = 60$) accession of *A. tequilana* Weber 'Azul' showed the highest total number of spines per leaf value (148), which is nearly 20% higher than in other polyploid accessions, but also nearly three-fold than that value observed in the also diploid *A. angustifolia* Haw. 'Marginata' (50); thus implying that different evolutionary pressures are in play in the diploid accessions and that the number of spines per leaf is characteristic of the species. Finally, taking together our results it is possible to suggest that in most *Agave* accessions polyploidy could be associated to morphological adaptations, which supports their capability for thriving in extreme environments.

3.2. Analysis of LEA and NBS-LRR genes and adaptation to stress

We analyzed 273 bp (~96aa) and 402 bp (~134aa) fragments corresponding to the domains LEA 3 and NB-ARC of the LEA and NBS-LRR genes, respectively. Translated amino acid sequences were highly conserved in all *Agave* accessions (>90%) (Fig. S4). The obtained sequences were submitted to the NCBI GenBank. Phylogenetic analyses were made comparing these gene fragments with previously isolated sequences from other angiosperm species (Battaglia et al., 2008; Chini et al., 2004; Loutre et al., 2009; Van Ooijen et al., 2008; Wise, 2003). NBS-LRR partial sequences were more closely related to the genus *Prunus* and to the species *Fragaria vesca* subsp. vesca and *Pyrus* × *bretschneideri*. Otherwise, those same sequences in the ancient angiosperm species of the genus *Solanum*, the family Brassicaceae (*Camelina sativa*, *Tarenaya hassleriana*, and *Eutrema salsugineum*), the tribe *Triticeae* (*Aegilops tauschii* and *Triticum aestivum*), and *Arabidopsis* were close to each other, but not too close to the NBS-LRR sequences of the accessions of *Agave* (Fig. 3). The LEA sequences of *Agave* accessions were more closely related to *Populus euphratica* and *Musa acuminate* subsp. Malacensis, but similarly to what was observed in the NBS-LRR gene sequences, no close relation was found with *Arabidopsis* and ancient angiosperms from the family Brassicaceae (*C. sativa*, *T. hassleriana*, and *Brassica rapa*) (Fig. 3). Because sequences of these genes for the family Asparagaceae were absent from the GenBank database, amino acid substitution analysis was only conducted by comparison of the LEA 3 and NB-ARC gene domains among sequences obtained in our study. This analysis indicated few amino acid substitutions in all *Agave* accessions (Fig. S4). Interestingly, diploid accessions of *A. tequilana* Weber ($2n = 2x = 60$) and *A. angustifo-*

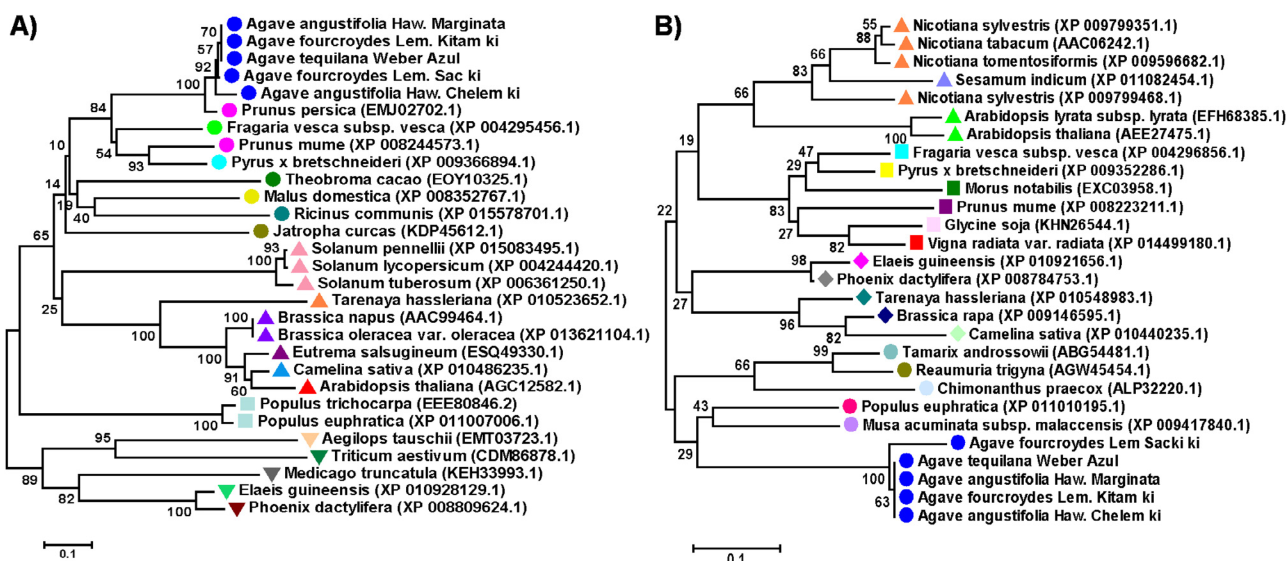

Fig. 3. Phylogenetic trees based on amino acid sequences of the NBS-LRR (**A**) and LEA (**B**) genes. Tree analysis was made by the neighbor-joining method with a bootstrap of 1000 replications. Numbers within parentheses indicate the protein accession numbers. Blue circles designate the sequences obtained in this study and each genus of plant is represented by a different colour or shape. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

lia Haw. 'Marginata ($2n = 2x = 60$) exhibited identical amino acid sequences, whereas the triploid accession of *A. fourcroydes* Lem. 'Kitam ki' ($2n = 3x = 90$) showed a single amino acid substitution in position 58 (serine by proline) in the LEA gene (Table S1). Contrastingly, polyploid accessions of *A. fourcroydes* Lem. 'Sac ki' ($2n = 5x = 150$) and *A. angustifolia* Haw. 'Chelem ki' ($2n = 6x = 180$) exhibited up to 10 amino acid substitutions in both genes (Table S1; Fig. S4) when compared with their lower ploidy counterparts. Most of these amino acid substitutions were non-synonymous mutations on the second nucleotide of different codons (Table S1), with 63% of transversions and 37% of transitions or deletions.

The *Agave* genomes we studied had a larger number of copies of the NBS-LRR genes than of the LEA genes with a maximum NBS-LRR/LEA ratio of 3000–1 and an average ratio of 2.5 (Table 2). The haploid genome of *A. tequilana* Weber 'Azul' ($2n = 2x = 60$) had 2.5 more copies of NBS-LRR gene than *A. angustifolia* Haw. 'Marginata ($2n = 2x = 60$), which appears atypical given that both accessions are diploid. Accessions of *A. fourcroydes* Lem. had in average 1.6 times more copies per haploid genome of the NBS-LRR gene (1157) than the same average for accessions of *A. angustifolia* Haw. (681.5). Each species had a different number of copies per haploid genome of the NBS-LRR gene, the largest numbers recorded in *A. tequilana* Weber 'Azul' ($2n = 2x = 60$), *A. fourcroydes* Lem. 'Sac ki' ($2n = 5x = 150$), and *A. angustifolia* Haw. 'Chelem ki' ($2n = 6x = 180$), the latter two accessions having the highest ploidy level recorded by us in those species.

Regarding the LEA gene, *A. tequilana* Weber 'Azul' ($2n = 2x = 60$) had 2 times more copies than the diploid *A. angustifolia* 'Marginata ($2n = 2x = 60$), similarly to what we observed in the copy number of the NBS-LRR gene. *A. tequilana* Weber 'Azul' ($2n = 2x = 60$) was the studied species of *Agave* having more copies per haploid genome of the gene LEA. In general, the average copy number of the LEA gene for all the analyzed species was of 423 and the higher such values were observed in accessions having a higher ploidy level within one same species, i.e., *A. fourcroydes* Lem. 'Sac ki' ($2n = 5x = 150$) and *A. angustifolia* Haw. 'Chelem ki' ($2n = 6x = 180$). Differences in the representativeness of these stress-response genes was supported by FISH analysis using LEA and NBS-LRR probes from a previously built BIBAC library (Tamayo-Ordóñez et al., 2012). This analysis allowed for a qualitative confirmation of the trend in copy number for both genes (Fig. S5 and S6), the accession of *A. tequilana* Weber 'Azul' ($2n = 2x = 60$) exhibiting the highest digoxigenin-FITC signals for both studied genes. Therefore, these results suggest a differential representativeness of these stress-related genes in this diploid *Agave* accession. Surprisingly, a single signal was observed regardless of the ploidy level of the analyzed species, which suggests that it is possible that these stress-related genes in *Agave* (CC-NBS-LRR and class 3 LEA) have the same chromosome organization in interphase nuclei.

Polyploidy is commonly recognized as having played a critical role in plant evolution and speciation (Wendel, 2000). The specific LEA and NBS-LRR genetic characterization observed in our study shows that polyploid accessions of *A. fourcroydes* Lem. 'Sac ki' ($2n = 5x = 150$) and *A. angustifolia* Haw. 'Chelem ki' ($2n = 6x = 180$) exhibit numerous (~10) amino acid substitutions (mostly transversions) compared to those observed in the studied diploid accessions of *Agave*. These amino acid substitutions have been described in polyploid plants as a consequence of functional specialization, subfunctionalization, neofunctionalization and pseudofuncionalization phenomena (Chen, 2007; Comai, 2005). The high rate of transversions (63%) we found could be related to the environmental characteristics of these plants' habitats. In this sense, it has been described that under exposition to ultraviolet light, *A. thaliana* Heynh in Holl & Heynh also shows an increased rate of transversions compared to transitions (Ossowski et al., 2010). The NBS-LRR and LEA genes described in plants are highly divergent (Chen, 2010; Trejo-calzada and O'Connell, 2005) and the primers designed in this study are only useful to amplify the 3 LEA and NB-ARC (nucleotide binding adaptor shared by NOD-LRR proteins, $APAF_{-1}$, R proteins and CED_4) domains of these genes. However, since we sequenced short partial regions of these genes it is possible that we amplified other members of the LEA and NBS-LRR gene families containing the same identified domains. For example, the NB-ARC domain is found in the NBS-LRR protein family and is coded by hundreds of diverse genes in each genome, which can be subdivided into the functionally distinct TIR-domain-containing (TNL) and CC-domain-containing (CNL). The gene we studied had higher identity values for type CC-NBS-LRR genes. In general, these large, abundant, proteins are involved in the detection of diverse pathogens, including bacteria, viruses, fungi, nematodes, insects and oomycetes, but their specific function remains unknown (Meyers et al., 2003; McHale et al., 2006; Wan et al., 2012). The genes belonging to the group 3 of LEA have two subgroups, 3A and 3B, the former repre-

Table 2
NBS-LRR and LEA gene copy numbers obtained by absolute real-time PCR (qPCR) in all studied accessions of *Agave* L.

Agave accessions	4C DNA content (pg)[a]	NBS-LRR gene		LEA gene		NBS-LRR/LEA rate
		Copy number per genome[b]	Copy number per haploid genome[c]	Copy number per genome[b]	copy number per haploid genome[c]	
A. tequilana Weber 'Azul' (2n = 2x = 60)	15.23	3,890.00 ± 126.00[d]	1,945.00 ± 63.00[D]	1,367.00 ± 113.00[a]	683.00 ± 56.00[B]	2.80
A. fourcroydes Lem. 'Kitam ki' (2n = 3x = 90)	22.56	3,224.00 ± 239.00[e]	1,074.00 ± 79.00[C]	1,305.00 ± 220.00[a]	435.00 ± 73.00[A]	2.40
A. fourcroydes Lem. 'Sac ki' (2n = 5x = 150)	37.46	6,207.00 ± 571.00[f]	1,241.00 ± 14.00[C]	2,209.00 ± 407.00[b]	441.00 ± 147.00[A]	2.80
A. angustifolia Haw. 'Marginata' (2n = 2x = 60)	n.d.	1,533.00 ± 941.00[g]	766.00 ± 470.00[A]	676.00 ± 158.00[c]	338.00 ± 79.00[A]	2.20
A. angustifolia Haw. 'Chelem ki' (2n = 6x = 180)	45.24	3,583.00 ± 353.00[e]	597.00 ± 58.00[B]	1,310.00 ± 339.00[a]	218.00 ± 56.00[A]	2.70

n.d. not detected.
[a] As reported by Robert et al. (2008).
[b] Lowercase letters (a–g) indicate significantly different values (Student's test; P > 0.05) of copy number per genome of each gene analyzed by qPCR. Values are averages of 3 replicates ± standard error.
[c] Capital letters (A–F) indicate significantly different values (Student's test; P > 0.05) of copy number per haploid genome of each gene analyzed by qPCR.

sented by D-7 LEA and the latter by D-29 LEA (Battaglia et al., 2008; Battaglia and Covarrubias, 2013; Dure et al., 1989; Dure, 2001). Interestingly, characterization of the group 3 LEA gene in *Zea mays* L. and *Citrullus lanatus* showed an accumulation of its transcripts under conditions of high salinity, low temperature, and osmotic and oxidative stress (Liu et al., 2013; Kim et al., 2015). Occurrence of these genes in *Agave* could be associated to a response to environmental stressing conditions such as high temperature, dehydration, and attack by pathogens, among others.

Despite the high diversity reported for the NBS-LRR and LEA genes (Battaglia et al., 2008; Battaglia and Covarrubias, 2013; McHale et al., 2006; Wan et al., 2012), it may be that in our work we failed to identify different alleles and isoforms within the same accession because of the short fragment of each gene analyzed by us and due to the coverage of the analyzed sequences being insufficient for detecting less represented alleles and isoforms. In experiments made by Tamayo-Ordoñez et al. (2015) using the same accessions of *Agave* and evaluating complete ribosomal genes, many haplotypes of the 5S rDNA genes, but more sequences and cloning events were used than in our present work. Therefore, more research must be conducted in order to identify the diversity of these genes within the genus *Agave*.

Otherwise, the classification and number of genes of the LEA and NBS-LRR gene families have been amply studied in *A. thaliana* Heynh in Holl & Heynh, *Oriza* spp., and other plants (Battaglia et al., 2008; Zhang et al., 2010), but nevertheless, determination of the number of copies of NBS-LRR and LEA genes present in plant genomes continues to advance, more such genes continuously being discovered. At present, more than 1600 NBS-LRR-type RGAs have been amplified via PCR from a wide range of plant species, and they have been arranged in clusters similar to R genes in plant genomes (Cannon et al., 2002; McHale et al., 2006; Wan et al., 2012). It seems as if these same genes are represented by thousands of copies and that they have a similar chromosome organization in the interphase nuclei of all the studied accessions.

The number of copies of the gene LEA has not been as thoroughly studied as that of the NBS-LRR gene. In *Arabidopsis*, 51 LEA genes have been described (Hundertmark and Hincha, 2008). We found in *Agave* that the LEA gene is represented by hundreds of copies, a number higher than that reported for other plant species, which is noteworthy given that the size of the genome of *Agave* is 33-fold that of *Arabidopsis* (Tamayo-Ordóñez et al., 2012). This difference in genome size could be a factor in the redundancy of copies and diversity of the LEA and NBS-LRR genes of *Agave*. The proportional number of copies of the genes NBS-LRR/LEA in the genomes of the studied accessions of *Agave* was similar to those determined by Tamayo-Ordóñez et al. (2012) by the characterization of a genomic BIBAC of *A. tequilana* Weber 'Azul' (2*n* = 2*x* = 60).

No previous reports exist for the genes we studied in *Agave*, however, by using capillary electrophoresis our research team demonstrated that the copies and haplotypes of these genes are represented in *Agave* by millions of copies per genome (Tamayo-Ordoñez et al., 2015), but although the number of copies was uncorrelated with ploidy level across accessions, polyploid species had more copies than their lower ploidy specific counterparts. In particular, no correlation was observed between gene copy number and ploidy level in *A. tequilana* Weber 'Azul', suggesting that in that species evolution of these genes may have been affected by the processes of artificial selection of productive characteristics carried out in the species (Tamayo-Ordoñez et al., 2015); a trend similar to that we observed in the present research in the number of NBS-LRR and LEA genes in *A. tequilana* Weber 'Azul'.

The observed trends of number of gene copies per genome in accessions of *Agave* can be the result of many factors, among which could be: the evolutionary mechanism of each analyzed gene, the origin and level of ploidy in each analyzed accession, artificial selection of productive characteristics, and environmental parameters. Dealing with the genus *Agave* is complicated due to their putative allopolyploid origin (i.e., resulting from intraspecific or intraspecific hybridization of one or more genomes), its relatively recent origin (ca. 8 M years ago), and the genus' high species diversification index relative to angiosperms in general (0.32 to 0.56 and 0.089–0.077 species per million years, respectively) (Good-Avila et al., 2006; Magallon and Sanderson, 2001; McKain et al., 2012). We suggest that, similarly to what has been observed in allopolyploids of *Nicotiana tabacum* (Kovarik et al., 2008, 2010; Tamayo-Ordoñez et al., 2015), the genes of *Agave* are undergoing an evolutionary process of fixation of the better gene copies, therefore being subject to several genetic transformations such as: recombination, amplification, duplication, transposition, gene loss, and genomic organization. For that reason, it is not surprising to find in *Agave* more copies of the NBS-LRR and LEA genes than in the paleopolyploid *Arabidopsis* in which processes of whole-genome duplication (WGD), massive gene loss, and genomic reorganization have already been completed (Blanc et al., 2003; Vision et al., 2000).

Otherwise, only in accessions of *A. fourcroydes* Lem. and *A. angustifolia* Haw. did the copy number of the LEA and NBS-LRR genes increased according to their ploidy levels. It is known that polyploidization could be associated with an increase in gene copy number and, therefore, involved in the generation of redundant genes (Lee and Chen, 2001). Specifically, a relation between rDNA gene redundancy and ploidy level had previously been observed in species of the genus *Agave* (Tamayo-Ordoñez et al., 2015). Gene redundancy in polyploid plant species can contribute to their better adaptation to habitats affected by climatic fluctuations and nutrient availability in the soil (Brochmann et al., 2004 Stebbins, 1971). Because of the various genetic events affecting the genes of

allopolyploid *Agave* accessions—derived from the genomic shock due to genome hybridization, we expected a non-lineal relation of number of gene copies and ploidy level across species, but we did expect that in accessions of different ploidy levels the number of gene copies increment and would be directly proportional to ploidy level, trends corroborated in our research, particularly in accessions of *A. fourcroydes* and *A. angustifolia*.

However, analysis of the accession of *A. tequilana* Weber 'Azul' ($2n = 2x = 60$), showed an atypical profile of the LEA and NBS-LRR gene representativeness, particularly for the latter gene. The variations in copy number exhibited by the NBS-LRR gene in plants can be the consequence of several factors including gene interaction, genome size, polyploidization, natural selection, and artificial selection (Hyten et al., 2006; Zhang et al., 2010). *A. tequilana* Weber 'Azul' ($2n = 2x = 60$), had the highest number of copies of the NBS-LRR gene of all studied accessions of *Agave*. In this sense, this diploid accession has proven to be most affected by pathogens in comparison to other *Agave* species (Gil-Vega et al., 2001). Therefore, an increment in NBS-LRR copies could result from gene amplification in response to infection by pathogens (Moreau-Mhiri et al., 1996, Vernhettes et al., 1997), or from the vegetative or *in vitro* culture propagation involved in its breeding (De-la-Peña et al., 2012; Díaz-Martínez et al., 2012). In this regard, the cultivated species *Oryza sativa spp. indica* has shown to express larger number of NBS-LRR gene copies when compared to its wild donor species (*Oryza rufipogon*), which suggests that crop domestication, breeding and cultivation have contributed to the size variations of the NBS and RLK families (Zhang et al., 2010).

Finally, it should not be a surprise that by means of FISH analysis only a single signal of the LEA and NBS-LRR genes is detected in all studied accessions of *Agave* and regardless of ploidy level, given that gene organization in interphase nuclei of polyploid species may differ (presence of one or more fluorescent signals) and it may or not be related to the ploidy level of the plant species (Leitch et al., 1992; Leitch, 2000; Bourdon et al., 2012; Heslop-Harrison et al., 1993). Many factors affect interphase chromosome organization such as histone modification and DNA methylation patterns, (Santos et al., 2011), changes in the ploidy level (Berr and Schubert, 2007), the shape and size of the nucleus (Berr and Schubert, 2007; Dittmer et al., 2007) during plant development and in response to the environment, as demonstrated in *Arabidopsis* (Tessadori et al., 2007) and *O. sativa* (Santos et al., 2011).

The study of interphase nuclear organization and the distribution of chromatin has been addressed for genes organized in tandem (rDNA) (Leitch et al., 1992; Walling et al., 2006), repetitive regions (satellite, centromeric, and telomeric) (Devi et al., 2005; Rawlins et al., 1991), but such studies have not been focused in the stress related genes NBS-LRR and LEA. The signals observed in FISH experiments in *Agave* are related to the location and organization of genes in chromosomes. Observing only one signal in FISH in all analyzed species, accessions, and ploidy levels could be indicating that the analyzed genes – related to responses to abiotic and biotic factors – share the same chromosome organization in interphase nuclei and are possibly located in a single locus.

In the *Arabidopsis* model the NBS-LRR and LEA genes have been shown to be subject of genetic changes from duplication, deletion, and other processes (Baumgarten et al., 2003; Hundertmark and Hincha, 2008; Jupe et al., 2012), which may originate new types of NBS-LRR and LEA genes, causing gene rearrangements (Leister et al., 1998). Such gene rearrangements may be mediated by unequal exchange of DNA fragments, gene conversions between homologous and homeologous chromosomes, and retrotransposition. In addition, it has been observed that in the plant species *Solanum tuberosum*, *A. thaliana*, and *Lotus japonicus* the CC-NBS-LRR and LEA type genes are amply distributed in the chromosomes and not in a single locus (Wise, 2003; Hundertmark and Hincha, 2008; Jupe et al., 2012; Marone et al., 2013; Li et al., 2010). But commonly genes belonging to a given gene type or gene class are located in a single locus, therefore it can be suggested that the organization in a single locus of the NBS-LRR and LEA genes in *Agave* (observed in FISH as one signal) can be due to us mapping only one single gene family for both genes (i.e., CC-NBS-LRR and LEA 3). The organization in clusters of genes related to a specific function has been amply discussed in eukaryotes (Lee and Sonnhammer, 2003). The organization of genes for a specific function in independent loci has been demonstrated to be due to gene divergence within clusters (Hurst et al., 2004). In *Agave* for example, it has been determined that despite the genes 45S rDNA and 5S rDNA are related to protein synthesis, they are located in completely independent loci (Robert et al., 2008). In addition, Garcia et al. (2010) stated that the organization and clustering of rDNA genes in the family *Asparagaceae* is not independent on the taxon, but could be the product of the evolutionary mechanism of these genes and to the genetic events they are undergoing, and in *Agave* Gomez-Rodriguez et al. (2013) demonstrated that the 18s rDNA locus is constant across ploidy levels, but the 5s DNA region is increased in diploid species, which may be due to an unequal exchange or transposition event, and suggesting that tandem organization of related genes can be directly related to gene divergence, gene function, and gene regulation mechanisms, and can be affected by all genetic factors to which they have been exposed. While these factors have a closer relation with chromosome organization, genes will have the same organization in the genome of species, and that organization may or not be related to the species' ploidy level. This suggests that despite the divergence in the analyzed multifamily genes in *Agave*, they possibly continue to share similar functions and regulation mechanisms allowing them for organizing in a single loci. A better understanding of the organization of genes in interphase nuclei may be of aid for gaining insight into the regulation, organization, and function of the LEA and NBS-LRR genes, which at present are unknown in *Agave*.

3.3. Expression analysis of the LEA and NBS-LRR genes

It has been observed that some polyploid plants are more tolerant to biotic or abiotic stresses in comparison to their diploid counterparts (Hao et al., 2013; Li et al., 2009; Meng et al., 2009; Shafieizargar et al., 2013; Wang et al., 2013; Yang et al., 2014). Some morphological and physiological characteristics may be associated to responses to abiotic and biotic stress in polyploidy plants (Doheny-Adams et al., 2012; Frank et al., 2011; Gutschick, 1999; Tamayo-Ordoñez et al., 2016; War et al., 2012).

In the case of our study, previous results suggested that some morphological characteristics could be associated to the ploidy level of studied *Agave* accessions (Fig. 2). Therefore, we also evaluated the genetic stress response of these *Agave* accessions by means of analyzing two stress-related genes (LEA and NBS-LRR) in different stress-induced conditions. We decided to incorporate the transcriptional analysis by two methods: relative RT-PCR, and absolute RT-qPCR.

The relative expression response of the abiotic stress-related gene LEA under saline and heat stresses showed that *A. tequilana* Weber 'Azul' ($2n = 2x = 60$), *A. angustifolia* Haw. 'Marginata' ($2n = 2x = 60$), *A. fourcroydes* Lem. 'Kitam ki' ($2n = 3x = 90$), and *A. angustifolia* Haw. 'Chelem ki' ($2n = 6x = 180$) regulate transcription of the LEA gene after 15 and 30 days of exposition to saline stress (induced by supplying 250 mM of NaCl) (Table S2). Initially, after 15 days of treatment, the average values of relative expression displayed were of 24.2, while 15 days later (30 day treatment) this same relative expression was reduced by 61.2%. However, *A. fourcroydes* Lem. 'Sac ki' ($2n = 5x = 150$) showed no significant differences in relative expression during exposition to saline stress.

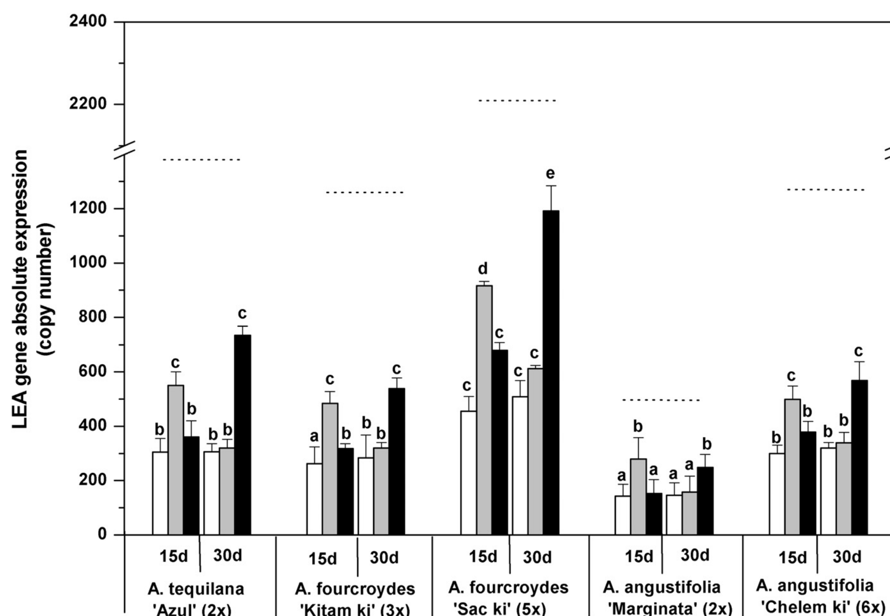

Fig. 4. Absolute expression of the LEA gene determined by RT-qPCR analysis during abiotic stressful conditions at 15 and 30 days of culture. Dotted lines indicate the representativeness (copy number) of the gene for each accession of *Agave* L. LEA gene expression is showed under optimal greenhouse (white bars), under salinity stress (grey bars), and under temperature stress (black bars) conditions. All values are averages of three replicates ± SE.

The relative expression of the LEA gene under heat stress (plants incubated at 40 °C) showed an inverse regulation relative to that observed during exposition to saline stress. During the first 15 days of treatment, the accessions with higher ploidy levels *A. fourcroydes* Lem. 'Sac ki' (2*n* = 5*x* = 150) and *A. angustifolia* Haw. 'Chelem ki' (2*n* = 6*x* = 180) showed the higher values of relative expression, followed by *A. fourcroydes* Lem. 'Kitam ki' (2*n* = 3*x* = 90), *A. tequilana* Weber 'Azul' (2*n* = 2*x* = 60), and finally, by *A. angustifolia* Haw. 'Marginata' (2*n* = 2*x* = 60). Interestingly, all *Agave* accessions showed significant differences after 15 days of exposure to heat stress, increasing their relative expression from 6 to 27 times, the highest values of relative expression corresponding to those from accessions of *A. tequilana* Weber 'Azul' (2*n* = 2*x* = 60) and *A. fourcroydes* Lem. 'Sac ki' (2*n* = 5*x* = 150) (Table S2).

Regarding the relative expression of the NBS-LRR gene, our results found significant differences only between *A. tequilana* Weber 'Azul' (2*n* = 2*x* = 60) and the other studied accessions (Table S2). This diploid accession had about 13 times more relative expression of NBS-LRR compared to the remaining studied accessions. In general, the studied *Agave* accessions showed no significant differences in relative expression after between 0.5, 6 and 12 h of infection.

The analysis of absolute expression RT-qPCR (copies number expressed) showed the response of the abiotic stress-related gene LEA under induced saline and heat stress conditions relative to the negative controls (plants adapted to unstressful conditions in the greenhouse). In all studied accessions of *Agave*, the expression profile of the LEA gene was mainly induced after 30 days of heat stress conditions. The above-mentioned expression response was higher in the polyploid accessions *A. fourcroydes* Lem. 'Sac ki' (2*n* = 5*x* = 150) and *A. angustifolia* Haw. 'Chelem ki' (2*n* = 6*x* = 180) compared to their lower ploidy level counterparts *A. fourcroydes* Lem. 'Kitam ki' (2*n* = 3*x* = 90) and *A. angustifolia* Haw. 'Marginata' (2*n* = 2*x* = 60). Surprisingly, the diploid accession of *A. tequilana* Weber 'Azul' (2*n* = 2*x* = 60) expressed 2.9 times less copies of the LEA gene than the diploid accession of *A. angustifolia* Haw. 'Marginata' (2*n* = 2*x* = 60) (Fig. 4). Saline stress also caused a LEA expression response, although at a lower proportion and faster (at 15 day) than the same under heat stress conditions. Once again, accessions with the highest ploidy level expressed more copies of the LEA gene than their lower ploidy level counterparts. This stress response expression of LEA was reduced in all *Agave* accessions after 30 days of assay. These differential responses of the LEA gene can be exemplified by *A. fourcroydes* Lem. 'Sac ki' (2*n* = 5*x* = 150) in which saline stress induced a faster accumulation of LEA transcript at 15 days, with 916 gene transcripts—a level that is 50% lower than that observed in optimal conditions. However, at 30 days of assay, accumulated LEA transcripts were reduced in 34%, reaching similar levels to those observed in optimal conditions.

With respect to the absolute expression response (expressed copy number) of the biotic stress-related gene NBS-LRR after infection with *E. carotovorum* (smith) Yabuuch in general and in all *Agave* accessions, the expression profile of this gene was mainly induced at the beginning of the infective process (0.5 h). The NBS-LRR gene transcript accumulation suddenly decayed at 12 h to similar levels than those in uninfected conditions (Fig. 5). Similarly to that observed in the LEA gene, this expression response was higher in the polyploid accessions *A. fourcroydes* Lem. 'Sac ki' (2*n* = 5*x* = 150) and *A. angustifolia* Haw. 'Chelem ki' (2*n* = 6*x* = 180), and in the diploid accession *A. tequilana* Weber 'Azul' (2*n* = 2*x* = 60). After 0.5 h of infection, accessions of *A. fourcroydes* Lem. 'Sac ki' (2*n* = 5*x* = 150) and *A. angustifolia* Haw. 'Chelem ki' (2*n* = 6*x* = 180) exhibited 75% and 60% higher copy number (transcript accumulation) of the NBS-LRR gene in comparison to *A. angustifolia* Haw. 'Marginata' (2*n* = 2*x* = 60) and *A. fourcroydes* Lem. 'Kitam ki' (2*n* = 3*x* = 90) counterparts, respectively. The accession of *A. tequilana* Weber 'Azul' (2*n* = 2*x* = 60) had the highest transcript accumulation of NBS-LRR gene with up to 1520 copies at 0.5 h after infection.

Interestingly, higher transcript induction of LEA or NBS-LRR was obtained in *A. fourcroydes* Lem. 'Sac ki' (2*n* = 5*x* = 150), *A. angustifolia* Haw. 'Chelem ki' (2*n* = 6*x* = 180), and *A. tequilana* Weber 'Azul' (2*n* = 2*x* = 60), which were the accessions having the higher LEA gene representativeness (Table 2). *A. fourcroydes* Lem. 'Sac ki' (2*n* = 5*x* = 150) and *A. angustifolia* Haw. 'Chelem ki' (2*n* = 6*x* = 180) exhibited the highest ploidy numbers, which supports the assumption that these polyploid *Agave* accessions are more tolerant to abiotic stresses in comparison to their 2*n* = 2x = 60 and 2*n* = 3*x* = 90 counterparts. However, the accession of *A. tequilana* Weber 'Azul'

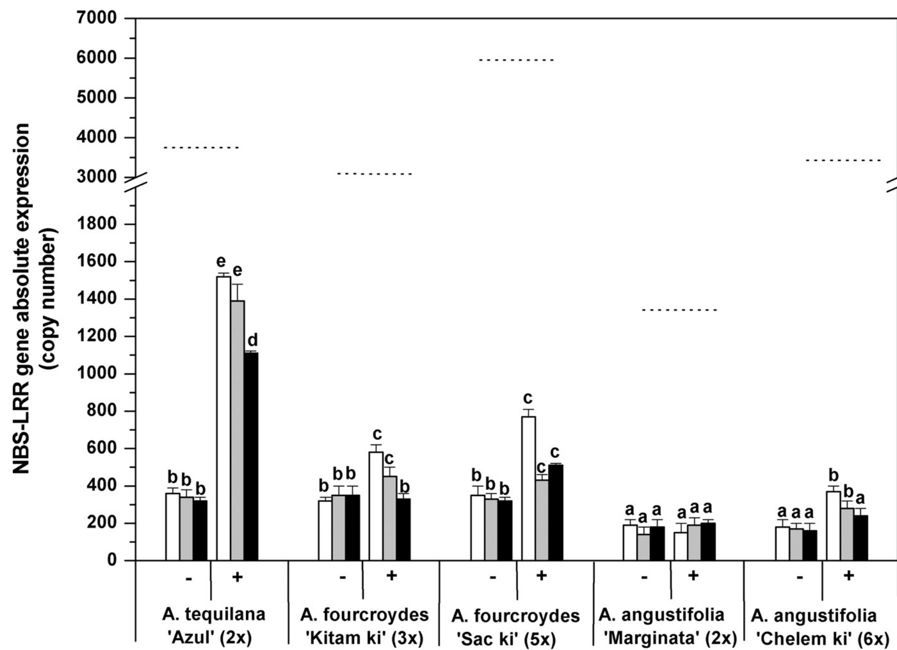

Fig. 5. Absolute gene expression of the NBS-LRR gene evaluated by RT-qPCR at 0.5, 5 and 12 h after pathogenic infection with *Erwinia carotovora* (smith) Yabuuch (+) Explants infected with *Erwinia carotovora* (smith) Yabuuch (−) Uninfected explants. Dotted lines indicate the representativeness (copy number) of this gene for each accession of *Agave* L. All values are averages of three replicates ± SE.

($2n = 2x = 60$) had also a high LEA or NBS-LRR gene response, which suggests a strong stress tolerance regardless of its diploid genomic level.

In general, despite we observed a higher level of expression of the LEA and NBS-LRR genes in higher ploidy level accessions, the number of transcribed copies and the relative levels of expression we detected were not proportional to the species' ploidy levels.

The partitioning of functions and the differences in expression patterns of these redundant genes are associated to physiological and phenotypic changes (Allario et al., 2011; Aversano et al., 2013; Chen 2007; Stupar et al., 2007) needed for supporting these advantages in allopolyploid plants. LEA and NBS-LRR genes are widely associated to physiological responses to stresses such as pathogenic infection, salinity, and temperature in diverse plant species (Arya et al., 2014; Chini et al., 2004; Dalal et al., 2009; Liang et al., 2013; Savitri et al., 2013; Selvaraj et al., 2013). In our study, LEA and NBS-LRR gene transcripts accumulated during abiotic (heat and saline) and biotic (pathogenic infection) stressful conditions, respectively. The observed differences in expression of the LEA and NBS-LRR genes among the species and ploidy levels we analyzed could have been related to the physiological, morphological, and molecular characteristics related to adaptation to stress in these plants, and that would be dependent on many factors, among which ploidy level is included. Studies aimed at identifying changes in genome expression patterns in allopolyploids are numerous, and it has been suggested that, unlike in autopolyploids (Allario et al., 2011), the expression of homeologous genes in allopolyploids may be regulated by divergent and regulatory elements associated with different subgenomes (Stupar et al., 2007; Rapp et al., 2009). It has been proposed that he genus *Agave* has allopolyploid genomes (McKain et al., 2012), which further complicates the regulation of genes represented by a large number of copies (such as LEA and NBS-LRR), causing an increase in the genome of both the gene copy number and the number of transcribed gene copies that is not proportional to ploidy level because of the genetic and epigenetic changes to which genes can be subject to due to genome hybridization.

Two reports exist of accumulation of LEA and NBS-LRR genes transcript, one of Martínez-Hernández et al. (2010) in several tissues of *A. tequilana* Weber 'Azul' ($2n = 2x = 60$), and a second one of Shakeel et al. (2013) during heat stress conditions in *A. americana*, but ours is the first study evaluating in *Agave* the expression of the NBS-LRR gene in response to attack by *E. carotovorum*. Vidal et al. (1997) and Glazebrook (2005) related the molecular response in presence of the pathogen to the JA/ET dependent signals pathways. Recently, it was demonstrated that this pathogen harbors the hairpin-encoding *hrp*N gene, which is an elicitor of the hypersensitive response (HR) (Rantakari et al., 2001; Ponce de León and Montesano, 2013), and that the virulence determinants hairpin (HrpN) and polygalacturonase (PehA) purified from *E. carotovora* subsp. *carotovora* can activate both the SA-dependent and JA/ET-dependent defense pathways (Kariola et al., 2003). These findings demonstrate the possibility of the increment in transcribed copy number of the genes NBS-LRR observed in *Agave* is the result of synergy between the SA-dependent and the JA/ET-dependent signaling pathways, same as it has been amply reported for the *Arabidopsis* model (Chini et al., 2004; Lawton et al., 1994; Thomma et al., 2001; Xu et al., 1994). In that sense, our research opens the way to further studies in *Agave* for understanding the role played by the NBS-LRR genes in response to the elicitors present in *E. carotovorum*.

Otherwise, the genus *Agave* exhibits adaptations allowing it to inhabit regions with severely stressful environments, and to tolerate throughout their life cycles the heat-limited, water-limited, and CO_2-limited environments of arid and semiarid habitats (Nobel, 1976; Nobel and Hartsock, 1978, 1979; Pimienta-Barrios et al., 2005a,b; Shakeel et al., 2013). The LEA and NBS-LRR expression responses we observed in this study were higher in the polyploid accessions of *A. fourcroydes* Lem. 'Sac ki' ($2n = 5x = 150$) and *A. angustifolia* Haw. 'Chelem ki' ($2n = 6x = 180$) and in the diploid accession of *A. tequilana* Weber 'Azul' ($2n = 2x = 60$). In this regard, it has been observed that some polyploid plants can better tolerate abiotic stress in comparison to their diploid counterparts (Hao et al., 2013; Li et al., 2009; Meng et al., 2009; Shafieizargar et al., 2013; Wang et al., 2013; Yang et al., 2014; Tamayo-Ordoñez et al., 2016). To this regard it is important to mention that of all the

Agave accessions we studied, the diploid accession of *A. tequilana* Weber 'Azul' ($2n = 2x = 60$) had the highest LEA or NBS-LRR transcript accumulation in response to stress induction. Although we did not compare the *A. tequilana* Weber 'Azul' ($2n = 2x = 60$) accession with a polyploid counterpart, its transcript accumulation and gene representativeness profiles differed from those in all other analyzed *Agave* accessions from other species. The observed LEA or NBS-LRR transcriptional differences in *A. tequilana* Weber 'Azul' ($2n = 2x = 60$) may be due to a disruption in gene regulation, which could also be affected by its intensive cultivation, vegetative propagation, and *in vitro* culture as was reported by Zhang et al. (2010) for other crop plant species in the genera *Oryza* spp. L. and *Gossypium* spp. L. (De-la-Peña et al., 2012; Díaz-Martínez et al., 2012; Kaeppler and Philips, 1993).

Finally, it is relevant to mention that our report is the first to use real-time qPCR for quantifying the number of copies and total expression of biotic and abiotic stress genes in complex plant genomes. Our methodology can be useful as a tool for future studies of complex plant genomes and for knowing the factors involved in gene redundancy, and gene expression profiles in polyploid genomes.

4. Conclusion

In conclusion, the *Agave* accessions we studied exhibited nuclear, stomatal and spine morphological values closely related with their corresponding ploidy levels. In addition, the polyploid accessions *A. fourcroydes* Lem. 'Sac ki' ($2n = 5x = 150$) and *A. angustifolia* Haw. 'Chelem ki' ($2n = 6x = 180$) had higher LEA and NBS-LRR gene representativeness, and a higher transcript accumulation under stress conditions when compared to their triploid or diploid counterparts. Under stress-inducing conditions and comparing all studied accessions, the diploid accession *A. tequilana* Weber 'Azul' ($2n = 2x = 60$) exhibited a differential stress-response profile with more spines per leaf, higher LEA and NBS-LRR gene representativeness, and higher LEA and NBS-LRR transcript accumulation. The singular gene expression observed in diploid *A. tequilana* Weber 'Azul' ($2n = 2x = 60$) suggests a differential evolutionary pressure is acting upon this species.

Our results also indicated that each accession analyzed has phenotypic differences that, together with the genetic background and transcript accumulation of genes related to stress (NBS-LRR and LEA), could help these species to better respond to environmental stress, also revealing the complexity of mechanisms present in polyploid *Agave* accessions.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jplph.2016.03.009.

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