RESEARCH ARTICLE

Response of transgenic tobacco overexpressing the *CchGLP* gene to cadmium and aluminium: phenotypic and microRNAs expression changes

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Abstract Transgenic tobacco (*N. tabacum* cv. Xanthi nc) expressing *Capsicum chinense CchGLP* gene that encodes an Mn-SOD, constitutively produces hydrogen peroxide that increase endogenous ROS levels. Previous studies using these plants against geminivirus infections as well as drought stress confirmed that *CchGLP* expression conferred resistance against biotic and abiotic stresses. Cadmium (Cd) and Aluminium (Al) contamination in soils are a major ecological concern since they are two of the most widespread toxic elements in terrestrial environments. Trying to explore additional possible tolerance to another stresses in these plants, the aim of this work was to analyse the response to cadmium and aluminium salts during germination and early stages of plantlet development and a differential transcriptome of microRNAs (miRNAs) expression in expressing *CchGLP* transgenic lines and an

azygote non-*CchGLP* expressing line. Plants were grown in vitro with addition of CdCl₂ and AlCl₃ at three different concentrations: 100, 300 and 500 µM and 50, 150 and 300 µM, respectively. The results showed higher tolerance to Cd and Al salts evaluated in two *CchGLP*-expressing transgenic lines L8 and L26 in comparison with the azygous non-*CchGLP* expressing line L1. Interestingly, L8 under Al stress presented vigorous roots and development of radicular hairs in comparison with azygous control (L1). Differentially expressed miRNAs in the comparison between L8 and L1 were associated with up and down-regulation of target genes related with structural molecule activity and ribosome constituents, as well as down-regulation in proton-transporting V-type ATPase (Vacuolar ATPase or V-ATPase). Moreover, KEGG analysis of the target genes for the differentially expressed miRNAs, led to identification of genes related with metabolic pathways and biosynthesis of secondary metabolites. One possible explanation of the tolerance to Cd and Al displayed in the transgenic tobaccos evaluated, might involve the fact that several down-regulated miRNAs, were found associated with target genes expressing V-ATPase. Specifically, miR7904-5p was down regulated and related with the up-regulation of one V-ATPase. The expression levels of these genes was confirmed by qRT-PCR assays, thus suggesting that a cation transport activity driven by the V-ATPases-dependent proton motive force, might significantly contribute as one mechanism for Cd and Al detoxification by vacuolar compartmentation in these transgenic tobacco plants.

Diana Sáenz-de la O and Christopher Alexis Cedillo-Jimenez have contributed equally to this work.

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Introduction

The development of tolerant crops represents an alternative to ensure agricultural production in contaminated regions. Cadmium (Cd) and Aluminium (Al) contamination in soils are a major ecological concern since they are two of most widespread toxic elements in terrestrial environments (Greco et al. 2012; Kochian et al. 2004). Cd is a non-essential element for plants, which is detrimental to all species even at very low concentrations. Its absorption is carried out easily by plant roots and translocated to the aerial organs causing chlorosis, leaf roll and inhibition of growth by affecting the processes such as photosynthesis, respiration and water and nutrient uptake (Greco et al. 2012; Yue et al. 2016). Cd-induced effects also include oxidative stress, inhibition of root metabolism and genotoxicity (Andresen and Hendrik 2013). Experiments related to Al stress reported a wide variety of effects, and they show that changes in root morphology and root growth inhibition are the primary consequences under Al stress in plants (Kochian et al. 2005). Al-induced effects include swollen root apices, root thickening, poor or no root-hair development, disturbances of root peripheral tissues and initiation of lateral roots closer to the root tip, altering the water balance and nutrient assimilation (Ciamporova 2002; Delhaize and Ryan 1995; Kochian et al. 2005).

Under stress, plants respond at the transcriptional and post-transcriptional levels to cope with the adverse conditions (Sunkar 2010; Cardenas-Manríquez et al. 2016). Changes in the levels of microRNAs (miRNAs) have been studied under different stress conditions in several plant species. These altered expressions have been found under abiotic stress conditions such as drought, salinity and heavy metal pollution (Shriram et al. 2016). Small RNAs (sRNAs), including miRNAs and short-interfering RNA (siRNA) are important in the regulation of diverse biological processes. Comparative studies of sRNAs from plants have mainly focused on miRNA, even though they constitute a fraction of the total sRNA diversity.

In previous work in our group, *Nicotiana tabacum* Xanthi nc expressing the gene *CchGLP* was engineered as an expression model for studying the elements involved in molecular defence networks in plants (Guevara-Olvera et al. 2012; Cardenas-Manríquez et al. 2016, 2018). The *CchGLP* gene, which was identified and characterized from a collection of habanero chilli peppers (*Capsicum chinense* Jacq BG-3821), codes for a germin-like protein (GLP) which showed Mn-superoxide dismutase activity (Mn-SOD) when expressed in *E. coli* (Barrera-Pacheco et al. 2008; León-Galván et al. 2011). GLPs have been associated with a variety of specific stages of plant development but particularly with different types of biotic and abiotic

stresses (Davidson et al. 2009; Dunwell et al. 2008). In fact, the aforementioned transgenic tobacco has shown tolerance to geminivirus infections, as well as drought stress (Guevara-Olvera et al. 2012; Cardenas-Manríquez et al. 2016). This tolerance correlated with the *CchGLP* expression, as well as the significant higher endogenous hydrogen peroxide levels in these plants in comparison to azygous non-*CchGLP* expressing controls and wild type tobacco (Guevara-Olvera et al. 2012). The metabolomic, proteomic and mRNAs transcriptomic profiles of these transgenic tobacco have already been reported (Cardenas-Manríquez et al. 2016, 2018). Based on the aforementioned, the aim of this work was to analyse the response to cadmium and aluminium salts during germination and early stages of plantlet development and a differential transcriptome of microRNAs (miRNAs) expression in expressing *CchGLP* transgenic lines and an azygote non-*CchGLP* expressing line.

Materials and methods

Plant material and germination assay

Two highly expressing *CchGLP* transgenic lines of *N. tabacum* xanthi nc (L8 and L26) and an azygote non-*CchGLP* expressing line (L1), which was used as a negative control, were generated in previous work and seeds were obtained by self-pollination (Guevara-Olvera et al. 2012). Azygous line (L1) control was used as a negative control because it harbours the *CchGLP* gene but does not show detectable expression levels of the *CchGLP* transcript (Guevara-Olvera et al. 2012). Seeds were surface-sterilized by incubation for 1 min in 95% ethanol, 7 min incubation in 10% bleach and 0.1% Tween 20 solution and finally five washes with sterile distilled water in a laminar flow cabinet. Seeds were sown directly in 90-mm sterilized Petri dishes, containing 20–25 seeds each one, in Murashige-Skoog medium with addition of CdCl₂ and AlCl₃ at three different concentrations: 100, 300 and 500 µM and 50, 150 and 300 µM, respectively. Kanamycin was also added into the medium for selection of transgenic seeds at a concentration of 100 µg/mL (Guevara-Olvera et al. 2012). Seeds were kept at room temperature (22 °C) under a photoperiod of 16-h light/8-h dark. Each treatment was replicated three times.

Effect of Cd and Al on germination percentage and root length

Germinated seeds were recorded every day after sowing until no further radicle emergence was observed (day 7) and germination percentage was then calculated. At

16 days after sowing, 10 plants of each treatment were sampled for the determination of plantlet length using a digital vernier.

RNA extraction and sequencing

L1 and L8 plants were grown in vitro and leaf samples were taken when seedlings began to develop true leaves. RNA was extracted from these samples using the hybrid protocol of TRIzol[®] Reagent commercial (Invitrogen, Carlsbad, CA, USA) and SV Total RNA Isolation System (Promega, Madison, WI, USA). RNA (fraction enriched in sRNA) was sequenced in the Sequencing Laboratory of the Institute of Biotechnology of the UNAM, Cuernavaca, Morelos using Genome Analyzer II by Illumina.

Gene expression analysis

RNA was extracted using the hybrid protocol of TRIzol reagent abovementioned. The synthesis of cDNA was made using the ThermoScientific Maxima strand cDNA synthesis kit for RTqPCR kit (BioAdvanced Systems Co.). The cDNA was quantified by spectrophotometry in a Spectrophotometer (Hoch DR6000). Primers sets used for evaluation of gene expression of ATPase, V0 complex, subunit e1/e2 (Nitab 4.5_0002313g0070.1) were as following: Forward 5'-CCTTCCACTAATCTGTTACACC-3', Reverse 5'-CCAAAGCTCAGAGAAATGTCC-3'. Quantitative PCR conditions consisted of 40 cycles, alignment temperature of 51 °C, using 18S rRNA gene for standardization and normalization of the results (Guevara-Olvera et al. 2012). For miR-7904-5p, the primers set was designed according to the methodology reported by Czimmerer et al. (2013). The cDNA was synthesized from total RNA using the stem-loop primer 5'-GTTGGCTC TGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAA CTGAGAT-3'; then the PCR primers used were: Forward, 5'-GTTGGTTCATAAACTCTG-3', Universal reverse primer, 5'-GTGCAGGGTCCGAGGT-3'. Quantitative PCR with the former primers was carried out with 40 cycles and alignment temperature of 51 °C. All the samples were analyzed in triplicate with their respective control using SyberGreen mix as fluorophore in a thermocycler equipment to quantify by RT-qPCR (BIO-RAD C1000). The results were evaluated using $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

MiRNA identification and annotation

Adapters were removed from the raw reads using Cutadapt v1.15 (Martin 2011) under the parameters “-e 0.05 -O 10 -m 15”, then low quality reads were removed using Trimmomatic v0.36 (Bolger et al. 2014) with the parameters

“SLIDINGWINDOW:4:24 MINLEN:15”. The low complexity reads were discarded with the BBDuk script of the BBTools package (Bushnell 2015) using an average entropy cut-off of 0.25 and parameters “entropywindow = 50 entropyk = 5”. Infernal software (Nawrocki et al. 2009) and Rfam database (Kalvari et al. 2018) were used to eliminate the reads originated from rRNA, tRNA, snRNA and snoRNA. According to the length criterion proposed by Axtell and Meyers (2018), only sRNAs sequences with length of 20–24 nucleotides were considered as putative miRNAs. Homology annotation using miRbase version 22 (Kozomara and Griffiths-jones 2014) was conducted to identify conserved miRNAs. This identification was performed using the same approximation described by Caballero-pérez et al. (2017).

Determination of differential expression of miRNAs

Differential miRNA expression analysis was carried out in the R package edgeR v3.22.5 (Robinson et al. 2010), by using the exactTest function. The resulting *p* values were input into the *q* value function with parameters “fdr.level = 0.05” to obtain a FDR of 5%.

MiRNA target prediction

A custom R script using criteria target recognition described by Schwab et al. (2005) was used to identify potential targets from the putative miRNAs (novel and conserved). Briefly, one mismatch was tolerated in the region complementary to nucleotides 2–12 of the miRNA, but not at the cleavage site (nucleotides 10–11), and up to 4 mismatches, but not more than 2 in row was tolerated in the region complementary to nucleotides 13 onwards. In addition, an identity of more than 70% between target sequences and the corresponding miRNA was required. The 3'- and 5'-UTRs sequences of all genes in *N. tabacum* genome v4.5 (Edwards et al. 2017) was used for the prediction analysis. Pearson correlation analysis between target genes and the corresponding miRNA was performed using the cor.test function implemented in R. The gene expression data was taken from previous work (Cardenas-Manriquez et al. 2018). Normality test for logarithms values of mRNA and miRNA expression was performed using the Anderson–Darling approximation (Anderson and Darling 1954), in order to avoid undetermined values, one was added to the expression amounts.

Gene ontology and KEGG analysis of significant target genes

GO enrichment analysis was performed with R package clusterProfiler (Yu et al. 2012), selecting significant Gene

Ontologies categories with q value < 0.05 . While KEGG pathways enrichment was analyzed with the R function phyper for hypergeometric test and Benjamini–Hochber’s approach for p value correction. For all enrichment analyses, the input gene set consisted of the transcriptome gene list (Cardenas-Manríquez et al. 2016) as a reference background, the target gene list of the 507 down-expressed miRNAs, and the target gene list of the 1407 up-expressed miRNAs.

Results

Effect of Cd and Al on germination percentage and plantlet performance

The germination rate of transgenic lines L8 and L26 was higher than the azygote control (L1) under all of the three

different concentrations of Cd and differences were highly significant, especially for L8 (Fig. 1a). At day 16 after sowing, under the Cd concentration of 100 μM , the development of the transgenic plants L8 and L26 did not show symptoms of stress and some of the plants already had true leaves (Fig. 2). However, for L1 plants under the same concentration, damage was evident since growth was retarded and plantlet length was undeveloped compared with L28 and L26 (Fig. 2). However, under the 300 and 500 μM concentrations of CdCl_2 , the three transgenic plants showed symptoms of stress such as growth inhibition and darkening and malformation of the roots (Fig. 2). The damage was more severe for L1 plants under the 300 μM concentration, showing many rolled leaves and malformed root tips, and at the concentration of 500 μM , they showed the complete arrest of growth (Fig. 2). For L1, the inhibition of plant growth and development in germinated seedlings was evident under the three concentrations

Fig. 1 Percentage of seed germination in the 3 transgenic tobacco lines evaluated at day 7 of exposure to several concentrations of cadmium chloride (CdCl_2) (a) and aluminium chloride (AlCl_3) (b). Values are the mean of 10 replicates. Different letters among the bars in the treatments for each transgenic line in each panel indicates significant difference using Tukey’s test ($P = 0.05$)

Fig. 2 Plantlets length and appearance of transgenic lines L1, L8 and L26 at day 16 after sowing in Murashige and Skoog medium with different concentrations of Cadmium Chloride (100, 300 and 500 μM)

(100, 300 and 500 μM). At the end of the experiments (day 16 after sowing), plantlet length and appearance of L8 transgenic plants was significantly higher than that of L1 control plants under 100 and 500 μM concentrations of Cd salt (Fig. 2).

Results of germination rate under Al stress showed that L8 was the only transgenic line displaying tolerance with the highest germination rates under the different Al concentrations (Fig. 1b). On the other hand, L1 and L26 displayed a similar response in germination under Al presence (Fig. 1b). Further microscopic observation of root morphology in L1 and L8 plants grown at 100 μM and 300 μM of AlCl_3 was carried out (Fig. 3). No differences were detected under 100 μM between the two lines. However, at

300 μM , the roots of L1 showed tip malformations, swelling and null presence of root hairs (Fig. 3). Meanwhile, L8 roots under the same concentration (300 μM) were more vigorous than L1 roots and even showed the presence of root hairs when compared with L8 at 100 μM of AlCl_3 (Fig. 3).

Differential microRNA expression analysis between L1 and L8

Since L8 and L1 showed the highest and lowest performance in the developmental process of seedlings respectively, the differential profiles of miRNA expression between L8 and L1 through sRNA sequencing analysis was carried out, using 3 biological replicates for each sample. Raw data for L8 and L1 libraries were deposited in NCBI Short Read Archive (SRA) under accession PRJNA496532. Low quality, adaptor sequences, and reads originated from rRNA, tRNA, snRNA and snoRNA were removed and unique sRNA sequences for L8 and L1 samples were obtained as mentioned in “Materials and methods”. A total of 142,560 common sRNA sequences were found in both lines and the main length in both samples was 17–29 nucleotides, which is consistent with the common length distribution of sRNAs studies in plants (Wang et al. 2017). However, the length of sRNA peaked at 38 nucleotides in the L8 sample, probably these sequences belonged to medium sized ncRNAs (31–200 nucleotides) expression. Only the subgroup of sRNAs with length of 20–24 nucleotides was considered as putative miRNAs, and was considered for downstream analyses, which resulted in L1 + L8 dataset of 4,369,268 sequences (870,573 excluding repeated sequences). The data

Fig. 3 Root tips of transgenic lines L1 and L8 treated with AlCl_3 at 16 days post-sowing

processing workflow and the resulting sequences is presented in supplementary materials (S1).

Using the dataset of putative miRNAs sequences, a search for conserved miRNAs by using miRBase v22 database (Kozomara and Griffiths-Jones 2014) and 317 sequences with homologues were carried out. The differential expression of putative miRNAs between L8 and L1 tobacco lines was analysed to estimate statistical significance of miRNA expression change using the following criteria: fold change (L8/L1) of 2 ($\log_2 = \pm 1$) and False Discovery Rate of 5% ($FDR < 0.05$). This analysis resulted in 507 down-expressed miRNAs and 1407 up-expressed miRNAs, of which 3 and 20 were annotated using miRBase, respectively.

Target gene prediction of the differentially expressed miRNAs in transgenic tobacco

A custom R script with the parameters for target recognition in plants proposed by Schwab et al. (2005), to predict the potential targets of *N. tabacum* miRNAs was used. Performing the target recognition between the 870,573 unique putative miRNAs dataset against (a) *N. tabacum* genome v4.5 (Edwards et al. 2017) and (b) the previously reported L1 and L8 transcriptome (Cardenas-Manriquez et al. 2018): it was found that 12,357 and 8425 (0.97%), respectively to (a) and (b), are sequences of putative miRNAs with at least one potential target. From these, 48 and 7 were up- and down-expressed miRNAs, respectively. On the other hand, regarding the expression of their targets, 256 down-regulated genes and 31 up-regulated genes were found (Supplementary material S2). Since in plants, targets of miRNAs are repressed as result of mRNA destabilization and/or translational repression, the analysis focused on the miRNAs that showed an opposite sense in the fold change in respect to their targets (quadrants II and IV in S2). Thus, there were found 814 miRNAs highly expressed in L8 corresponding to down-regulated target expression in the transcriptome of the L8 and 278 miRNAs with an equivalent pattern in L1 plants. Their corresponding targets were 360 and 176 genes, respectively. From these 1092 miRNAs, 139 were observed with at least one target differentially expressed in the transcriptome, from which 98 miRNAs with high expression in L1 displayed a total of 38 targets with lower expression in the same plants, while the rest 41 miRNAs showed high expression and their corresponding 47 targets less expression in L8. In order to test the effect of the miRNA-mRNA interaction, a comparison of the transcriptional levels of gene targets and their corresponding miRNAs for each plant line separately detected showed distinct correlation patterns (Supplementary material S3).

GO and KEGG analysis of significant target genes

A functional category enrichment analysis of the target gene list of predicted miRNAs was performed using Gene Ontology (GO) and KEGG pathways information. Structural molecule activity (GO:0005198) and structural constituent of ribosome (GO:0003735) were significantly high in target gene sets of up-regulated miRNAs (Fig. 4a). While, ribosome (GO:0005840), structural molecule activity (GO:0005198), structural constituent of ribosome (GO:0003735), proton-transporting V-type ATPase complex (GO:0033176) and proton-transporting V-type ATPase V0 domain (GO:0033179) were significantly high in target gene sets of down-regulated miRNAs (Fig. 4b). In a similar analysis, the spliceosome and biosynthesis of secondary metabolites pathways for the target gene sets of down-regulated miRNAs was identified (Fig. 5b), and observed that DNA replication and repair related pathways in target gene sets of up-regulated miRNAs, such as nucleotide excision repair, DNA replication, base excision repair, homologous recombination, etc., as well as, ribosome biogenesis, spliceosome and biosynthesis of secondary metabolites pathways were also found (Fig. 5a).

Validation of gene expression in a miRNA-mRNA target pairwise related with heavy metals tolerance

In order to validate the gene expression of the pairwise miR-7904-5p (down-regulated in the bioinformatic analysis) and the corresponding target gene proton-transporting V-type ATPase V0 domain (up-regulated in the bioinformatic analysis), qRT-PCR assays were carried out. The results of relative gene expression confirmed that miR-7904-5p was down-regulated in L8 and up-regulated in L1 control, and the overexpression of the V-ATPase in L8 in comparison with L1 (Fig. 6).

Discussion

Effect of Cd and Al on germination percentage and plantlet performance

L8 resulted in higher percentage of germination and plantlet performance under the Cd and Al concentrations evaluated in this work in comparison with L26 and L1. Among them, transgenic L8 showed significantly the highest germination rates in the different concentrations (Fig. 3). Moreover, L8 plants displayed the best performance and even presented more lateral roots under Al stress while roots of control plants showed more damage, malformation and absence of lateral roots under stress treatment. These data suggested that root development and

Fig. 4 Statistically significant ontologies identified by term-enrichment of target genes of predicted miRNAs. Enriched target genes from **a** up-regulated miRNAs, **b** down-regulated miRNAs

morphology adaptations are required for heavy metal adaptation and that L8 have acquired the genetic machinery in response to Al stress. Zhu et al. (2015) found that wheat grains showed no differences in the radicle emergence time comparing with 5000 μM of Al comparing to free-salt control. In another study, application of nano-aluminium oxide concentration of 98 μM for 10 days in plants of *Arabidopsis thaliana* increased their photosynthetic activity and root weight and length (48% and 39%, respectively) compared with control but under 196 μM concentration, plants showed decreased shoot and root weight (Jin et al. 2017). These results together suggest that low concentration of Al tends to be beneficial for some species (as a possible hormetic effect) but that even under high concentrations of Al, some species (e.g. wheat and the tobacco L8 tested in the present study) have developed resistance and are able to survive and grow. From the differential miRNAs found, some down-regulated genes related with V-ATPases with roles as driving proton motive force were identified in the present study. This finding suggests that detoxification by compartmentation of Cd and Al by cation transportation to vacuoles, might be one of the mechanisms by which the transgenic tobacco L8 plants displayed tolerance to these heavy metals. V-ATPases have been demonstrated in several species such as cucumber and

barley to increase gene expression and transport activity when exposed to heavy metals (Dietz et al. 2001; Janicka-Russak et al. 2012). Moreover, it is important to mention that some differential miRNAs were found down-regulated and associated with target genes related to increase of secondary metabolites biosynthesis. This result is in concordance with previous reports with these transgenic tobaccos displaying that constitutively L8 plants have significant higher amounts of chlorogenic acid and rutin than L1 (Cardenas-Manríquez et al. 2016). This latter aspect suggests that these phenolic compounds might also display an antioxidant role to cope the oxidative stress caused by Cd and Al in the transgenic tobacco plants studied in this work. These two phenolics in plants such as maize have been reported to play a role in oxidative stress (Kisa et al. 2016; Sharma et al. 2019).

Interestingly, although both transgenic lines L8 and L26 highly express the transgene *CchGLP* and based on the response to Cd and Al salts in this work, they displayed differences in the tolerance to these metals' salts. Under the technology tested in this study, the transgenic events occurred randomly in the genome with a separate regulatory package related to each insertion. This suggests that the transgene acts depending on the site to which it has been inserted in the genome of the plant. At least under Cd

Fig. 5 Statistically significant pathways identified by KEGG pathway-enrichment of target genes of predicted miRNAs. Enriched target genes from **a** up-regulated miRNAs, **b** down-regulated miRNAs

Fig. 6 Relative gene expression of a pairwise microRNA and target gene related to Cd and Al tolerance in transgenic tobacco to *CchGLP* gene. **a** Gene expression of miR-7904-5p; **b** gene expression of V0-

ATPase. Bars in each graph indicates the standard deviation of three replicates of the analysis. For details of gene expression normalization, see “Materials and methods”

and Al stresses, L8 was more tolerant than L26; despite the *CchGLP* expression was similar (Guevara-Olvera et al. 2012). Similarly, in a study comparing the response under chilling stress between two transgenic tomato lines (homozygous for yeast S-adenosylmethionine decarboxylase) and their azygote control, results showed that the levels of the metabolites of interest that were studied differed between the transgenic lines (Goyal et al. 2016). Transgenic tobacco L8, has previously reported as tolerant to geminivirus infections and drought, both associated to the

high endogenous levels of H_2O_2 produced by the superoxide dismutase activity (SOD) by the *CchGLP* transgene (Guevara-Olvera et al. 2012; Cardenas-Manríquez et al. 2016). Additionally the mRNA transcriptome, metabolome and proteomic profiles of L8, indicates that the defense system in this plant is constitutively induced (Cardenas-Manríquez et al. 2016, 2018); thus, this aspect might explain the tolerance to Cd and Al salts displayed in the present research by L8 in comparison with the azygous control and the line 26.

Alteration on root architecture due to miRNA regulation and of secondary metabolism

The GO and KEGG analyses found mainly predicted targets of down-regulated miRNAs related to secondary metabolites biosynthesis, and up-regulated miRNAs related to DNA biosynthesis and repair (Figs. 4, 5). The former results suggested that the balance between the miRNAs expression and their respective target genes is mainly dedicated to produce secondary metabolites for defense, thus explaining at least partially, the observed tolerance to Al and Cd salts in this work for L8 and L26 tobacco lines. This aspect is in concordance with previous results that demonstrated the tolerance to geminivirus infections and drought stress of highly *CchGLP*-expressing transgenic tobacco lines as L8 and L26 evaluated in this work (Guevara-Olvera et al. 2012; Cardenas-Manríquez et al. 2016, 2018).

Among the structural molecule activity found in the GO analysis, a DnaJ-like protein was found as target of the sequence of an up-regulated miRNA in L8 (TCAAAC-GAGAAAGGCTTACGG). Lee et al. (2018) found that expression of DnaJ-like gene in alfalfa was induced by abiotic stress of chilling and heat but not by Cd. Moreover, Sofo et al. (2017) reported the reduction of root length, surface and reduction in lateral roots development under Cd stress in *A. thaliana*, probably due to auxin was localized in the apical at low levels. Strikingly, a molecular relation between auxin and DNAJ-like protein has been reported. Nakamura et al. (2019) reported that ALTERED RESPONSE TO GRAVITY1 (ARG1) encodes DNAJ-like protein and are related to root and hypocotyl regulation due to gravitropism that is influenced by a physical gradient of sedimentation on amyloplasts that trigger signal transduction for auxin transport direction. In the present work, it was found that under Cd stress, the L8 roots were larger and in Al stress, L8 root architecture resulted in more vigorous and with lateral roots than control (Fig. 3). Moreover, the L8 constitutive expression of *CchGLP* produces high levels of endogenous hydrogen peroxide in tobacco (Cardenas-Manríquez et al. 2018) and it was found that ectopic applications of hydrogen peroxide in *A. thaliana* caused amyloplast deficiency in columella cells, alteration in the polar expression of the Pin-formed 1 (PIN1) and PIN2 and, in consequence, translocation and distribution of auxin in a phenomenon of gravi-sensing loss (Zhou et al. 2018). These data suggested that auxin fluxes might be altered due to the endogenous production of hydrogen peroxide that under heavy metal stress might result in a physiological advantage. However further investigation of the interaction between the response of gravitropism and heavy metals regulated by miRNAs

through the targeting of DnaJ-like translation might be carried out.

Conclusions

Taking together the results of this work suggested that the expression of *CchGLP* in tobacco (*N. tabacum* cv. Xanthi nc) improved the tolerance to Al and Cd stress in early stages, and this phenotype was at least in part, related to miRNAs expression associated to biosynthesis of secondary metabolites as chlorogenic acid and rutin and overexpression of V-ATPases, likely used to cope with these stress conditions.

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