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Chemical inhibition of the histone acetyltransferase activity in *Arabidopsis thaliana*

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ABSTRACT

Chemical inhibition of chromatin regulators provides an effective approach to investigate the roles of chromatin modifications in plant and animals. In this work, chemical inhibition of the Arabidopsis histone acetyltransferase activity by γ-butyrolactone (MB-3), the inhibitor of the catalytic activity of mammalian GENERAL CONTROL NON-REPRESSIBLE 5 (GCN5) is evaluated. Arabidopsis seedlings were germinated in LS medium supplemented with different concentrations of MB-3, and inhibition in the root length and yellowed leaves were observed. The yellowed leaves phenotype of the plants grown in 100 μM of MB-3 was reverted when plants were additionally treated with 1 μM of TSA, a histone deacetylase inhibitor. Using an immunoblot assay with specific antibodies revealed a reduction of H3K14 acetylation levels at 3 and 24 h post-treatment. At 24 h post-treatment a reduction of H3K9 acetylation levels was observed. Targets of GCN5 related to stress were downregulated at 3 h post-treatment but no change was observed in target genes related to developmental transition. Our results indicate that MB-3 is a chemical inhibitor of the histone acetyltransferase in Arabidopsis and suggest that this inhibitor could function in other plants species.

1. Introduction

Histone acetylation is associated with transcriptional activation and plays important roles in numerous developmental and biological processes in eukaryotes [1]. Histone modification is executed by a family of histone acetyltransferase proteins (HAGs) highly conserved in eukaryotes. In plants, this family has been described in Arabidopsis [2], grape [3], rice [4] and tomato [5]. Among the different HAGs, GCN5 has been described as a subunit of the histone acetylation (HAT) module of SAGA complex in plants [6]. The GCN5 protein mainly modifies H3K14 in yeast [7] and Arabidopsis [8,9]. However, acetylation of histone H3K9 and H3K27 on target promoters is also affected by a *gcn5* mutation in Arabidopsis [8]. In addition, recent studies have indicated that GCN5 catalyzes the acetylation of H3K36 [10].

In Arabidopsis, GCN5 has important roles in numerous developmental and biological processes, including fatty acid biosynthesis [11], cellular patterning of root epidermis [12], thermotolerance [13], iron homeostasis [14], control of the juvenile-to-adult phase transition [15], inflorescence meristem and stamen development [16], root development [17], shoot development [18,19], light-responsive gene expression [8], response to low temperatures and salt [20,21], regulation of miRNA production [22], and auxin-induced transcription [23].

Chemical inhibition of epigenetic regulators provides a quick and effective approach to investigate the roles of epigenetic modifications in controlling many biological processes [24]. Compounds that inhibit the activity of enzymes involved in chromatin remodeling have been useful tools in elucidating mechanisms underlying epigenetic regulation in Arabidopsis. For example, 5-aza-2'-deoxycytidine (5-aza) is an analog of 5-cytosine that cannot be methylated and thereby inhibits DNA (5-cytosine) methylase, reducing the overall level of DNA methylation in chromatin [25]. In addition, Trichostatin A (TSA) is a broad-spectrum inhibitor of histone deacetylases (HDACs) and can be used to increase histone acetylation in chromatin [26]. Both compounds have been applied in plant epigenetics studies, especially in species for which genetic resources is limited [24].

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A chemical inhibitor of GCN5 was designed and evaluated in human cells [27]. This compound is a class of α-methylene-γ-butyrolactone (MB-3) and inhibits the catalytic activity of mammalian GCN5 *in vitro* [27]. The use of MB-3 in plants has been recently reported in Arabidopsis. Weiste and Dröge-Laser [23] reported significantly reduced auxin-induced transcription of marker genes when MB-3 was applied in the protoplast for 6 h. Moreover, Lee et al. [28] reported that the expression of circadian marker gene *CCA1* is reduced when 10-day-old seedlings were treated for 48 h with MB-3. While in both cases the authors assumed that MB-3 inhibits the catalytic activity of GCN5 *in planta,* there is no evidence of molecular and physiological effects of MB-3 in the whole plant.

In this work, the effect of MB-3 on the growth and inhibition of the catalytic activity of GCN5 in Arabidopsis seedlings were evaluated. We report that a treatment of MB-3 produces an inhibition of root growth and chlorotic leaves, which is associated with a reduction in histone acetylation and downregulation of some known direct targets of GCN5. Our results indicate that MB-3 is a chemical inhibitor of the histone acetyltransferase in Arabidopsis and suggest that this inhibitor could function in other plants species.

2. Materials and methods

2.1. Plant materials and growth conditions

Seeds of *Arabidopsis thaliana* (ecotype Columbia-0) were surface-sterilized and placed in Petri dishes containing Linsmaier and Skoog (LS) salts medium supplemented with 0.8% agar-agar. After cold treatment at 4 °C for 2 d, the seeds were transferred to 22 °C under a 16/8 h light/dark cycle in a growth chamber (Percival Scientific, Inc., Perry, IA, USA). For root length determinations, the lengths of roots from root tip to hypocotyl base were measured using the National Institutes of Health program ImageJ (Bethesda, MD, USA).

2.2. Chemical compounds

Trichostatin A (TSA, T8552), 5-aza-2′-deoxycytidine (5-aza, A3656), and γ-butyrolactone (MB-3, M2449) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All the compounds were prepared in DMSO.

2.3. Protein extraction and Western blot

A Histone-enriched extract was prepared with 12-day old Arabidopsis plants treated with 100 μM MB-3 or DMSO as control. 0.1 g of frozen tissue was ground and homogenized in extraction buffer (10 mM Tris pH 7.5, 2 mM EDTA, 0.4 M HCl, 5 mM beta-mercaptoethanol, 0,2 mM PMSF) and was incubated overnight at 4 °C with gentle rotation. The samples were centrifugated at 12000g for 10 min at 4 °C and the supernatant was incubated for 1 h on ice with TCA in a final concentration of 25% (p/v). The samples were centrifuged at 17000 g for 30 min at 4 °C and the pellet was washed twice with 100% acetone. The pellet was dried and then dissolved in 0.1 ml of distilled water supplemented with protease inhibitors. 15 μg of protein extract was used for SDS-PAGE, and Western blot was preformed with anti-histone H3 antibody, anti-histone H3 (Acetyl K9) antibody and anti-histone H3 (Acetyl K14) antibody (Abcam; ab1791, ab10812, ab52946, respectively). In addition, total proteins were extracted from Col0 plants treated with DMSO as control and 100 μM MB-3 at the indicated times. After the SDS-PAGE and transfer to a PVDF membrane the antibodies anti-histone H3, anti-histone H3 (Acetyl K9) and anti-histone H3 (Acetyl K14) (Abcam; ab1791, ab10812, ab52946, respectively) were used following the manufacturer's instructions.

2.4. Quantitative RT-PCR analyses

Total RNA was extracted using the PureLink RNA mini kit (Ambion, Life Technology, Carlsbad, USA) from 12-day-old seedlings treated with 100 μM MB-3 and controls at 3 h and 24 h after treatment. 1 μg of total RNA treated with DNAse I (RQ1, Promega, Madison, USA) was reverse transcribed with oligo-dT25 primer using Superscript II reverse transcriptase (Life Technology, Carlsbad, USA) according to the manufacturer's instructions. Quantitative RT-PCR was performed using the Brilliant® SYBR® Green QPCR Master Reagent Kit (Agilent Technologies, Santa Clara, USA) and the Eco Real-Time PCR detection system (Illumina®, San Diego, USA), as described by Poupin et al. [29]. The *CLATHRIN*, *SAND* (AT2G28390) and At4g26410 (unknown function) genes were used as internal controls. The relative expression level of each gene in MB-3 treatment was compared to control conditions and calculated as described previously by Matus et al. [30]. Normalization was performed using the CLATHRIN cDNA level and averaged over three replicates. qRT-PCR analyses were performed with two biological repeats. An unpaired *t*-test was calculated for each time point. The primers used are listed in Supporting Information Table S1.

3. Results

MB-3 has been demonstrated to efficiently target amino acids within the catalytic active site of the mammalian GCN5 enzyme [27]. A sequence alignment analysis using both the single HAT domain (Fig. 1) and the full predicted protein sequences (Fig. S1) of yeast, human, Arabidopsis, rice, and eucalyptus GCN5 homologous with the Arabidopsis members of the GNAT/MYST family (HAG2, HAG3 and HAG5) was produced to identify conserved catalytic amino acids. This analysis revealed that amino acids described to be crucial for HAT activity and targeted by the HAT inhibitor MB-3 are conserved in the GCN5 proteins and are not present in others HATs in Arabidopsis (Fig. 1), suggesting that MB-3 could be a specific inhibitor of GCN5 in plants.

To evaluate the effect of MB-3 on plant growth, Arabidopsis seedlings were germinated in LS medium supplemented with different concentrations of the GCN5 inhibitor (50, 100 and 150 μM). Fig. 2 illustrates the phenotype of seedlings recorded 12 days after sowing. No effects were observed at 50 μM, but reduction in root length paired with yellowed leaves, a phenotype easily recognized in *gcn5* mutants reported in the literature, was observed at 100 μM and 150 μM. The yellowed leaves phenotype of plants grown in 100 μM of MB-3 was reverted when plants were

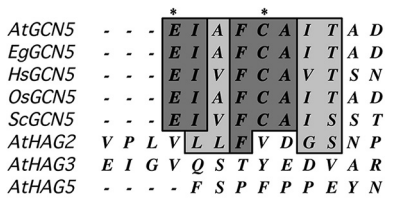

Fig. 1. Protein sequence alignment of the catalytic domain of histone acetyltransferases. The HAT domain was identified using the conserved domain tool of NCBI. The protein alignment was constructed with the clustal W method. Blocks highlighted in grey mark similar residues. The amino acids with asterisks have been described be crucial for HAT activity and are targeted by the chemical inhibitor MB-3. The protein accession numbers are: AtGCN5 (AT3G54610), EgGCN5 (XP_010038046), HsGCN5 (NP_066564), OsGCN5 (XP_015614202), ScGCN5 (NP_011768), AtHAG2 (AT5G56740), AtHAG3 (AT5G50320) and AtHAG5 (AT5G09740).

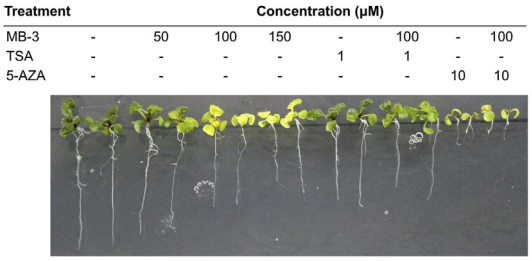

Fig. 2. Phenotype of seedlings treated with chemical inhibitors. Arabidopsis seedlings were germinated in different concentrations of MB-3, TSA and 5-AZA, as is indicated in the figure and the plant growth was visualized 12 d later.

additionally treated with 1 μM of TSA, a broad-spectrum histone deacetylase inhibitor, suggesting that TSA can antagonize the effect of MB-3 in leaves (Fig. 2). A broader view of shoot growth is shown in Fig. S2. The root length was quantified at the same time from the root tip to the base of the hypocotyl. Fig. S3 illustrates a significant reduction in the root length at 100 and 150 μM of MB-3. Treatment with TSA also inhibited root growth and masked the effect of MB-3 when both compounds were applied at the same time. No additive effect is observed when 100 μM MB-3 is treated with 10 μM 5-aza (Fig. 2 and S3).

Chemical inhibition facilitates studying the direct role of GCN5 in short-term treatments. Because phenotypic differences are not observed when the inhibitor is applied over short time frames (hours), we performed an immunoblot assay to confirm the GCN5 inhibitory activity of MB-3 in plants. A histone-enriched extract was prepared with 12-day old Arabidopsis plants treated for 3 h or 24 h with 100 μM MB-3 or DMSO as control, and the level of histone acetylation was analyzed using a specific antibody (Fig. 3). H3K14 acetylation levels were reduced by MB-3 treatment at 3 and 24 h. H3K9 acetylation levels were not affected at 3 h but a slight reduction could be observed at 24 h (Fig. 3). These results suggest that MB-3 works as a histone acetyltransferase inhibitor in Arabidopsis.

Histone acetylation is generally correlated with gene activation. In order to relate decreased acetylation of H3K14 with changes in gene expression, well-characterized genes that showed a down-regulation in the mutant *gcn5* were selected for expression analysis after short treatment with MB-3. The *NITRATE TRANSPORTER 1.5* (*NRT1.5*), *PROLINE TRANSPORTER 1* (*PROT1*), *CA2+ ACTIVATED OUTWARD RECTIFYING K+ CHANNEL 5* (*TPK5*), *COLD-RESPONSIVE 6.6* (*COR6.6*) and *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 3 and 9* (*SPL3* and SPL9) were selected [13,15]. and their expression patterns were measured by quantifying mRNA accumulation in whole wild-type plants at 3 and 24 h after MB-3 treatment. Quantitative RT-PCR results showed that no significant differences were observed in the expression levels of *SPL3* and *SPL9* in response to MB-3 treatment (Fig. 4). Conversely, the expression levels of *NRT1.5*, *PROT1*, and *TPK5* were down regulated in plants exposed for 3 h to the chemical inhibitor but were restored to control condition expression levels after 24 h of treatment. Interestingly, the expression of *COR6.6* was down-regulated 3 h after MB-3 treatment, but was up-regulated 24 h after the same treatment (Fig. 4).

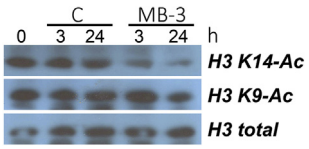

Fig. 3. Alteration of histone H3 acetylation levels during MB-3 treatment. Western blot showing H3K9ac and H3K14ac signal in histone-rich extract isolated from control and MB-3 treated Arabidopsis seedlings.

4. Discussion

In this study, we show physiological and molecular evidence for the inhibition of histone acetyltransferase activity in *Arabidopsis thaliana* by MB-3. This compound was designed as the first small-molecule inhibitor of human GCN5 [27]. MB-3 presents a possible hydrogen-bond acceptor for the backbone amide of C177 and a polar group for interaction with E173 [27], and both amino acids are conserved in the active site of HAT in homologous GCN5 proteins (Fig. 1). Interestingly, those amino acids are not present in other members of the GNAT/MYST family (HAG2, HAG3 and HAG5) of Arabidopsis, suggesting that MB-3 could be a specific inhibitor of GCN5 in plants. In humans, MB-3 inhibits GCN5 with an IC50 (The half maximal inhibitory concentration) value of 100 μM [27]. The affinity of MB-3 for GCN5 is comparable to that of the natural substrate H3 [27]. In this work, we determined that 100 μM of MB-3 is the minimum concentration that produces the maximum effect, significantly reducing the length of the root and inducing a change in leaf color (Fig. 2 and S3). Lee et al. [28] used the same concentration to evaluate the involvement of histone acetylation in the circadian expression of *CCA1* in Arabidopsis seedlings. The effect of MB-3 on Arabidopsis growth is masked by TSA, a non-specific inhibitor of histone deacetylation (Fig. 2). TSA treatment is associated with the hyperacetylation of histones H3 and H4 [31], suggesting that TSA has an opposite effect with respect to MB-3.

Histone Lysine acetylation is often positively associated with transcription and has important roles in numerous developmental and biological processes in plants. Among the different Histone acetyltransferase, the GCN5 protein mainly modifies H3K14, H3K9, H3K27 and H3K26 [10]. We analyzed the short-term effect of MB-3 on H3K14 and H3K9 histone modifications using specific antibodies (Fig. 3). At 3 h post-treatment, we observed a decrease in the acetylation of H3K14, which is maintained at 24 h. H3K9 acetylation is not affected at 3 h but a decrease is observed at 24 h (Fig. 3). Earley et al. [9] deduced that H3K14 acetylation is specific to GCN5 using a pre-acetylated peptide assay, demonstrating that an H3 peptide that had been previously acetylated on lysine 9 served as an excellent substrate for GCN5-catalyzed acetylation [9]. However, prior acetylation of H3 lysine 14 was shown to block radioactive acetylation of the peptide by GCN5 [9], indicating the substrate specificity. Our results suggest that MB-3 inhibits the histone acetyltransferase activity in plants. We do not discard the modifications of other histone markers due to indirect effects. Further experiments using specifics antibodies will reveal what happens downstream of histone acetyltransferase inhibition in histone modification and chromatin remodeling. Indeed, H3K14 acetylation by GCN5 is mechanistically coupled to H3 serine10 phosphorylation and is required for the expression of a subset of genes in yeast [32,33].

In order to evaluate the transcriptional effect of MB-3, we evaluated the expression of several known targets of GCN5 in 12 days-old seedlings during short-term treatments (Fig. 4). The expression levels of genes related to abiotic stress (*NRT1.5, PROT1, TPK5, COR6.6*) are down-regulated at 3 h post MB-3 treatment, but at 24 h the expression is restored to basal levels, with the exception of *COR6.6*, which is up-regulated at this time. *COR6.6* encodes a hydrophilic peptide that is induced by cold, ABA, drought, and salt stress [34], and its induction is correlated to an increase of H3 K14/K9 acetylation [35]. The significant induction of *COR 6.6* after 24 h of MB-3 treatment concomitant with a reduction in H3K14 and K9 acetylation requires clarification with further experiments. The expression levels of genes related to developmental transition

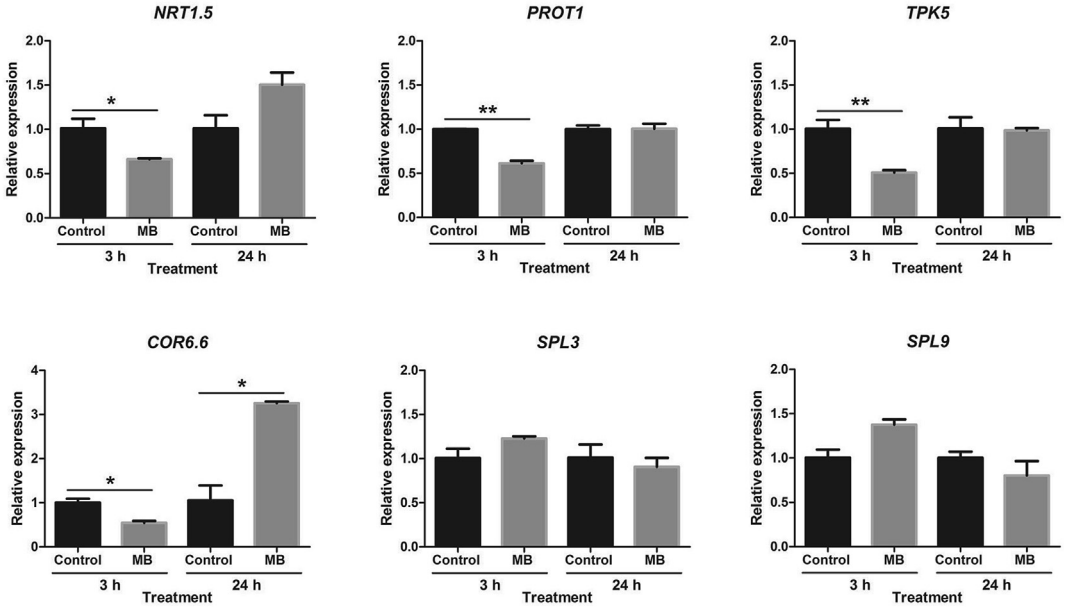

Fig. 4. Expression profiles of known target genes regulated by GCN5 in plants treated with MB-3. Relative levels of gene expression determined by quantitative RT-PCR in whole 12-day-old Arabidopsis control and treated with 100 μM of MB-3 at time indicated in the figure. Data are means ± SE. Asterisk indicates statistical significance (unpaired *t*-test, $^{*}p < 0.05$, $^{**}p < 0.01$). MB: MB-3.

(*SPL3* and *SPL9*) did not change because their expression patterns depend on the state of development of the plant and are controlled by conserved microRNAs of the miR156 family [36]. In our case, the plants used in this experiment were 12 days old, and the post-transcriptional regulation of SPLs is likely still in action.

In conclusion, our molecular and physiological results indicate that MB-3 is a chemical inhibitor of the histone acetyltransferase in Arabidopsis and suggest that this inhibitor could function in other plants species. The identification and application of chemical histone acetyltransferase inhibitors in plants is useful for the analysis of molecular and biological processes related to the crosstalk of histone modifications and chromatin remodeling.

Contributions

FA conceived the study and designed the experiments with TT and AH. FA, TT and AHV performed the experiments and analyzed the data. FA drafted the paper. All authors contributed to the revision of the manuscript and approved the final version.

Acknowledgment

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2016.12.086.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2016.12.086.

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