Research Article

Chaetocin enhances callus induction by decreasing the expression of major leaf polarity genes in *Nicotiana tabacum*

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Abstract: Using chemicals known as inhibitors of chromatin remodeling enzymes is a useful approach in understanding the regulation machinery that contributes to the maintenance of or conversion to a pluri- and totipotent states of the cells in multicellular organisms. They can serve as tools to reveal the molecular and cellular mechanisms required for reprogramming. A deeper understanding of reprogramming pathways that drive the cell into a dedifferentiated state might help us enhance plant micropropagation and green biotechnology applications. Chaetocin is one of the histone methyltransferase inhibitors known as key drivers in several cellular processes. It was shown that chaetocin enhanced the reprogramming of human fibroblasts. Here, by using an analogy approach, we tested the effects of chaetocin on the post-germination growth and callus induction in *Nicotiana tabacum* for the first time. We found that chaetocin retarded the leaf development in *N. tabacum* seedlings but increased the callus formation when applied in the post-germination growth phase (1.4 fold). The expression of *AS1*, *KAN1*, *YAB3*, *FIL*, major leaf polarity genes, significantly decreased when seeds were treated with Ch during the post-germination growth phase by 69, 45, 46, 68%, respectively. We also observed that chaetocin could not replace 2,4-Dichlorophenoxyacetic acid as a callus inducing modulator. The present study is a pioneer, exhibiting valuable data to understand the molecular mechanisms underlying the effects of chaetocin and the unknown properties of histone-modifying enzymes. Hence, novel approaches can be developed by using high throughput methods to elucidate epigenetic mechanisms of cellular differentiation/dedifferentiation processes in plant systems.

Key words: Tobacco, chromatin modifiers, post-germination growth, dedifferentiation

1. Introduction

In eukaryotic cells, epigenetic mechanisms including DNA methylation, chromatin remodeling and histone post-translational modifications (PTMs) are the key components to determine the 3-Dimensional (3D) structure of highly organized nucleoprotein complex named chromatin (Liu et al., 2010; Hajheidari et al., 2019). While the maintenance of identities of stem cells and differentiated cells in plants is related to the stability of epigenetic marks, the transition from stem cells to somatic cell types, induction of pluripotency in differentiated cells, and response to environmental stresses are represented by the reversibility of epigenetic changes (Verdeil et al., 2007; Ahmad et al., 2010; Iwasaki and Paszkowski, 2014; de la Paz Sanchez et al., 2015). The delicate balance in these critical processes and alteration of cell fate are cooperatively orchestrated by epigenetic regulators guided by certain physiological and environmental conditions. (Berenguer et al., 2017; Inácio et al., 2018; Zhang et al., 2018; Singh et al., 2020). Studies to understand the action mechanism of epigenetic regulators in various biological processes and manipulate them for biotechnological purposes have come into prominence in recent years (Berenguer et al., 2017; Yu et al., 2017; Vaijayanthi et al., 2018; Ruta et al., 2019).

One of those vital processes in plant life is post-embryonic development. In the post-embryonic developmental phase, some cells remain in a less-differentiated stage as in meristems; shoot apical meristem (SAM) and root apical meristem (RAM) which are the origin of all above- and below-ground organs, respectively (Yadav et al., 2013; Gaillochet and Lohmann, 2015). The maintenance of stem cells is the main reason for post-embryonic development, providing organ regeneration when a plant is exposed to a wide range of environmental changes, including herbivore and pathogen attacks throughout its life, and for reprogramming them into different plant parts at the tissue culture level (Gaillochet and Lohmann, 2015; Sang et al., 2018; Warghat et al., 2018). In SAM, stem cells characterized by *Wuschel* (*WUS*), *Clavata 3* (*CLV3*), and *Knotted-1-* like homeobox (*KNOX*) genes are located in the central zone (CZ) (Bowman and Eshed, 2000; Gaillochet and Lohmann, 2015). The cell fate transition from peripheral zone (PZ) of SAM to leaf primordium is promoted by expression of leaf abaxial-adaxial polarity genes including *YABBY3* (*YAB3*), *FILAMENTOUS FLOWER* (*FIL*), *KANADI 1* (*KAN1*), and *ASYMMETRIC LEAVES 1* (*AS1*) genes (Byrne et al., 2002; Kumaran et al., 2002; Sarojam et al., 2010). Especially, repression of *KNOX* genes by *FIL* and *YAB3* is essential for the initiation of the cellular differentiation process to form leaves (Kumaran et al., 2002). It has been shown that multiple mutations on leaf polarity genes give rise to ectopic expression of *KNOX* and *WUS* genes that allow SAM structure to re-establish and thereby promote the loss of leaf identity (Kumaran et al., 2002; Eshed et al., 2004; Sarojam et al., 2010; Palermo and Dornelas, 2020). Besides transcriptional regulation, post-translational modifications (PTMs), such as methylation, acetylation, and phosphorylation of the protein tails of the core histones, are

critical for controlling gene expression in plant development (Liu et al., 2010; Berr et al., 2011; Singh et al., 2020). However, the epigenetic regulation pathway in the maintenance of stem cells and cellular differentiation during plant development needs to be detailed (Ruta et al., 2019; Cheng et al., 2020; Su et al., 2020).

The methylation of lysine (K) residues of Histone 3 (H3) and Histone 4 (H4) are the best-studied PTMs and are known as key drivers in several cellular processes (Pontvianne et al., 2010; Bannister and Kouzarides, 2011; Cheng et al., 2020). H3 core is particularly modified by methylations on K4, K9, K27, and K36, which are associated with transcriptional activation (H3K4 and H3K36) or repression (H3K27 and H3K9) by histone lysine methyltransferases (HKMTs) (Liu et al., 2010; Zhou et al., 2020). Three protein groups of HKMT enzymes, Suppressor of variegation 3-9 [SU(var) 3-9], Enhancer of zeste [E(z)], Trithorax (trx) are also named as SET domain group (SDG) proteins (Jenuwein et al., 1998). These proteins have SET domains that are conserved evolutionarily in the wide range of eukaryotic organisms and can transfer the methyl groups to target amino acids (Zhou et al., 2020). SDG proteins are involved in regulating plant developmental plasticity, cellular reprogramming, and response to environmental stresses (Pontvianne et al., 2010; Thorstensen et al., 2011; Zhou et al., 2020). The Polycomb-repressive complex 2 (PRC2) is one of the Polycomb-group (PcG) histone lysine methyltransferases, which contains the E(z) subfamily and is responsible for targeting gene repression through performing H3K27 trimethylation (Ruta et al., 2019; Yu et al., 2019). PRC2 protein plays a central role in epigenetic control of the cell, tissue, and organ fate assignation during plant development (Liu et al., 2010; Bannister and Kouzarides, 2011; Mozgova et al., 2015). It has been shown that the specific inhibitor of the PRC2 in animal systems, 1,5-bis (3- bromo-4-methoxyphenyl) 1,4-pentadien-3-one (RDS 3434), induces epigenetic changes in *Arabidopsis* seedlings by decreasing H3K27me3 level and affects root growth (Valente et al., 2012; Ruta et al., 2019).

The methylation of lysine 9 of histone H3 (H3K9) performed by SUV group HKMTs has a repressive effect on gene expression like PRC2 mark, H3K27me3 (Cheng et al., 2005; Pontvianne et al., 2010). There are seven subclasses of the SUV subfamily and two clusters: SUV homologs (SUVH) and SUV-related homologs (SUVR) in plants (Pontvianne et al., 2010; Zhou et al., 2020). However, two protein groups have different additional domains and can transfer different numbers of methyl groups to H3K9 residues (Cheng et al., 2005; Johnson et al., 2007). SUVH and SUVR subfamilies play a central role in epigenetic control of many developmental processes and repression of transposons in plants (Pontvianne et al., 2010; Zhou et al., 2020). The euchromatic G9a methyltransferase has a crucial role in the formation of H3K9 methylation in early embryogenesis and is a major target in epigenetic therapy of several types of cancer (Tachibana et al., 2002; Casciello et al., 2015). The G9a SET domain showed significant similarity with the plant SUVR4 enzyme (Baumbusch et al., 2001; Thorstensen et al., 2006). Since BIX-01294 is an inhibitor of the G9a enzyme and leads

to reduce H3K9me2 levels in several mammalian cell lines, it can be utilized to understand the mechanism of HKMTs actions in different cellular processes (Chang et al., 2009; Huang et al., 2016; Li et al., 2020). It has been tested that BIX-01294 affects microspore reprogramming into embryogenesis; at the early stages, BIX-01294 induced cell reprogramming, totipotency and initiation of embryogenesis by reducing H3K9 methylation level (Berenguer et al., 2017).

Chaetocin (Ch), a fungal mycotoxin, was reported as the inhibitor of lysine-specific histone methyltransferases such as SUV39H1, which catalyzes H3K9 di- to trimethylation, and G9a (Greiner et al., 2005). Ch-based inhibition of SUV39H1 has been shown to be a promising therapeutic approach for several human cancer types and improved developmental competence through epigenetic reprogramming of cloned embryos (Lai et al., 2015; Weng et al., 2019). Onder and his colleagues (2012) shown that the suppression of SUV39H1 by small hairpin RNA (shRNA) enhanced somatic cell reprogramming in dH1fs (derived from H1 human embryonic stem cell fibroblasts) around 1.8-fold, and it was shown that Ch enhanced reprogramming of dH1fs around 1.2-fold as well (Ebrahimi et al., 2019).

As mentioned above, histone-modifying enzymes (histone modifiers, HMs) direct several cellular processes, including differentiation during post-embryonic development, stem cell maintenance, cell reprogramming and phenotypic plasticity to environmental conditions through determining the 3-D structure of chromatin in plants (Bannister and Kouzarides, 2011; Zhou et al., 2020). Although the tremendous differences in the form of life and cellular construction in animals and plants, epigenetic regulators share significant gene and functional homology. The evolutionarily conserved SET domains contained in the HKMTs have a central role in controlling gene expression in both kingdoms (Pontvianne et al., 2010; Zhou et al., 2020). Thus, the epigenetic inhibitors proved to affect the SET domain of HKMTs in animal systems can be a promising approach for understanding the action of epigenetic regulators in several cellular processes also in plants (Li et al., 2014; Berenguer et al., 2017; Ruta et al., 2019).

This study aims to evaluate the effects of chaetocin on the post-germination growth and dedifferentiation process in tissue culture and its use as a possible epigenetic modulator in plants for the first time. Therefore, initially, we applied Ch during seedling growth on a model plant organism, *Nicotiana tabacum*. The leaves of seedlings grown in the presence of Ch were tested for callus induction, and qRT-PCR analysis was performed for major leaf polarity genes (*YAB3, FIL, KAN1, AS1*) in tobacco plantlet.

2. Materials and methods

2.1. Seed sterilization and germination
Nicotiana tabacum seeds were provided by Ege Agricultural Research Institute. The seeds were soaked in 70% EtOH for 2-3 min and later 2.3% sodium hypochlorite solution (commercial bleach) for about 30 min for sterilization and washed three times via sterile water to remove EtOH and NaClO. Sterilized seeds were planted in solid basal MS (Murashige and Skoog, 1962) medium. For the preparation of

MS medium, 4.4 g MS salt (Sigma-Aldrich Corp., M5519, St. Louis, MO, USA) and 30 g sucrose (Sigma-Aldrich, 84097) were dispersed in 800 mL distilled water, then distilled water was added until the solution became 1 L. Later on, pH value was adjusted to 5.7 by 1N NaOH, then 8 g agar was added (Sigma-Aldrich Corp., A-1296, St. Louis, MO, USA) to the medium. After autoclaving, 2 mL of 0.22 μm membrane-filtered MS vitamin (Sigma-Aldrich, M3900) was added. Thirty tobacco seeds were placed on solid MS medium in each 6 cm Petri dish and incubated in the growth chamber (Heraeus, Vötsch, No: 440/0026/86) at 25 ± 2 °C under 16/8 h of photoperiod at 1400 lux. A total of 4–7 days later, dormant seeds were eliminated from the culture dishes.

2.2. Growth conditions

Chaetocin (Ch, Sigma, C9492) was diluted with dimethyl sulfoxide (DMSO, Sigma-Aldrich Corp., D8418, St. Louis, MO, USA) to reach a final concentration of 1 mg/mL and stored at –80 °C for further use. According to Ebrahimi (2016), 5 μM Ch was reported as a non-toxic dose for the treatment in the germination of *Nicotiana tabacum* seedling. Therefore, 5 μM Ch was used in the experiments. Germinated seeds were transferred to solid MS medium containing 5 μM Ch and to MS medium used as control w/o Ch. For both groups, 7 germinated seeds were placed on 6 cm-Petri dishes for further development. Petri dishes were incubated for 1 month in the growth chamber at the same culture conditions, as described above. At the end of one month, seedlings (5 μM Ch-treated and control) were used for RNA isolation.

2.3. Callus induction

Leaves of 1 month old seedlings were used as explants for callus induction. Leaf discs were cut out by a cork-borer. Then, callus induction and Ch treatment experiments were done as described in Table 1. 2,4-Dichlorophenoxyacetic acid (2,4-D, Sigma-Aldrich Corp., D-7299, St. Louis, MO, USA), a synthetic auxin, was used for callus induction. Four leaf discs were placed on each 6-cm Petri dish in solid MS media containing 0.5 mg/L of 2,4-D, and explants were incubated at the same culture conditions of plant growth, as described previously. After one month of incubation, the number of generated calli were counted, and the dimensions were measured by a ruler.

In addition, we designed time course callus induction experiments to understand the role of this small molecule during post-germination growth and whether it can induce callus formation in the absence of 2,4-D (Table 1). Leaf discs from plantlets, treated with 5 μM Ch during post-germination growth were incubated in only-Ch-containing medium without using any synthetic auxin (2,4-D) for one month for callus formation (Table 1, 4th condition).

2.4. RNA extraction and cDNA synthesis

Based on the results of time-course experiments of callus induction, total RNA was isolated from the leaves of 1 month old Ch-treated (2nd condition, Table 1) and of control groups to further analyze the expression levels of the major genes involved in leaf polarity (Table 2). The leaf samples were frozen with liquid nitrogen and homogenized for isolation by grounding with a mortar and pestle. TRIzol (Invitrogen, 15596026) was used for RNA extraction, and 1 μg of isolated RNA sample was used for cDNA synthesis by Proto Script II First Strand cDNA Synthesis Kit (NEB, E6300) according to the kits' procedures.

2.5. Quantitative real-time analysis (qRT-PCR)

RT-PCR analysis was performed using the SensiFAST SYBR No-ROX Kit (Bioline, BIO-98020). Primer3 (primer3.ut.ee8) and NCBI Primer Blast software was used to design the qRT-PCR oligonucleotide primers for *AS1*, *KAN1*, *YAB3*, and *FIL* target genes, which are responsible for leaf formation in *N. tabacum*. Primers were designed according to the length of each primer, melting temperature, GC% content, and size of the PCR product. Designed primers were synthesized by Sentromer DNA Technologies company (İstanbul, Turkey). Gene expression levels were calculated by using the ΔΔCt method and normalized to the 18S rRNA housekeeping gene as a reference. The list of target genes and primer sequence pairs are given in Table 2.

2.6. Statistical analysis

Post-germination growth and callus induction experiments were done in 10 biological and 3 technical replicates. qRT-

Table 2. RT-PCR primers for target genes in *N. tabacum*

Target genes	Forward primer sequences (3'–5')	Reverse primer sequences (3'–5')
18S	CTATTGGAGCTGGAATTACCG	GGCTACCACATCCAAGGAA
AS1	ACTCGCCTTAACCTCCAAGC	ACCTGAAAATGCGCCCCTTA
KAN1	AACACCTTTAAGCCTCGGGG	AGAGGCATCCCGGTCTATCA
YAB3	TAGCCTTCTTCGTGGGAGGA	GGCAACCCTGATATCAGCCA
FIL	GTGTATGTGGGGGAAGTGGG	AACCGCCCTCCAGAGAAAAG

PCR experiments were done in 2 biological and 2 technical

Table 1. The experimental plan and time course treatment of Ch on *N. tabacum*. 30th day of germination was taken as day zero (0), and the leaves of 30 days old seedlings were used for callus induction experiments.

Period and stages Experimental conditions	Day: -30 ◄────► 0 Post-germination growth	Day: 0 ◄────► 30 Callus induction (Dedifferentiation)
Control	MS	MS + 0.5 mg/L 2,4-D
1st condition	MS + 5μM Ch	MS + 0.5 mg/L 2,4-D + 5μM Ch
2nd condition	MS + 5μM Ch	MS + 0.5 mg/L 2,4-D
3rd condition	MS	MS + 0.5 mg/L 2,4-D + 5μM Ch
4th condition	MS + 5μM Ch	MS + 5μM Ch

replicates. The mean values and their standard deviation of all experiments were calculated. A two-tailed student T-test was performed to indicate the significance of the data, and the data with a p-value ≤ 0.05 were evaluated as significant.

3. Results

3.1. Effects of chaetocin on the leaf growth of *N. tabacum*

After incubating the seeds in MS (control) and 5 μM Ch-containing mediums for one month, it was observed that the Ch-treated plantlets had significantly smaller and yellowish leaves than the control group (Figure 1a and 1b).

3.2. Chaetocin enhances callus induction of N. tabacum

The number and size of the calli coming from leaf discs of 30 days old plantlets grown on 5 μM Ch-treated groups showed 12% and 20% enhancement compared to the control, respectively. These results were shown statistically significant (Figure 2).

According to the results of time-course experiments, the application of Ch at a concentration of 5 μM at the post-germination growth stage significantly increased callus formation compared to control (Figure 3a). It was found that this increase was 1.4 times more than the control and was statistically significant (Figure 3d and 3f). As it can be seen from the Figure 3b-e, it was found that Ch treatment during callus development (3rd condition) did not cause any improvement and even had a reducing effect in callus induction compared to the control groups (Figure 3e).

3.3. Chaetocin cannot induce callus formation in the absence of 2,4-D

After 5 μM Ch treatment in both post-germination growth and callus induction phases (4th condition in Table 1), no callus development was observed at the end of culturing

period. This finding showed that Ch cannot be used instead of 2,4-D for callus induction in tobacco (Figures 4a and 4b).

3.4. Effect of the chaetocin treatment on the expression levels of leaf differentiation genes

The results of the time course treatment showed that only Ch-treatment in the post-germination growth phase (2nd condition) had a significant effect on callus induction. Therefore, qRT-PCR analyses were performed using only the leaf materials sampled from these plantlets, cultured in MS w & w/o Ch.
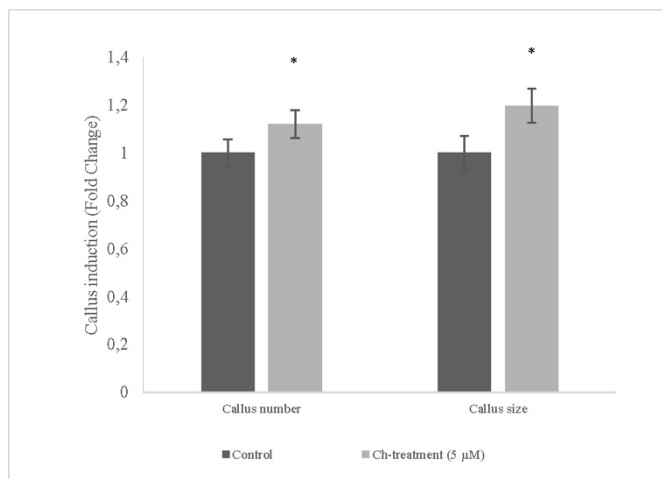

Figure 2. Effects of Ch treatment on the number and size of the generated calli at day 30 of culturing with w/o 5 μM Ch. Error bars = standard deviation. *p-value ≤ 0.05.
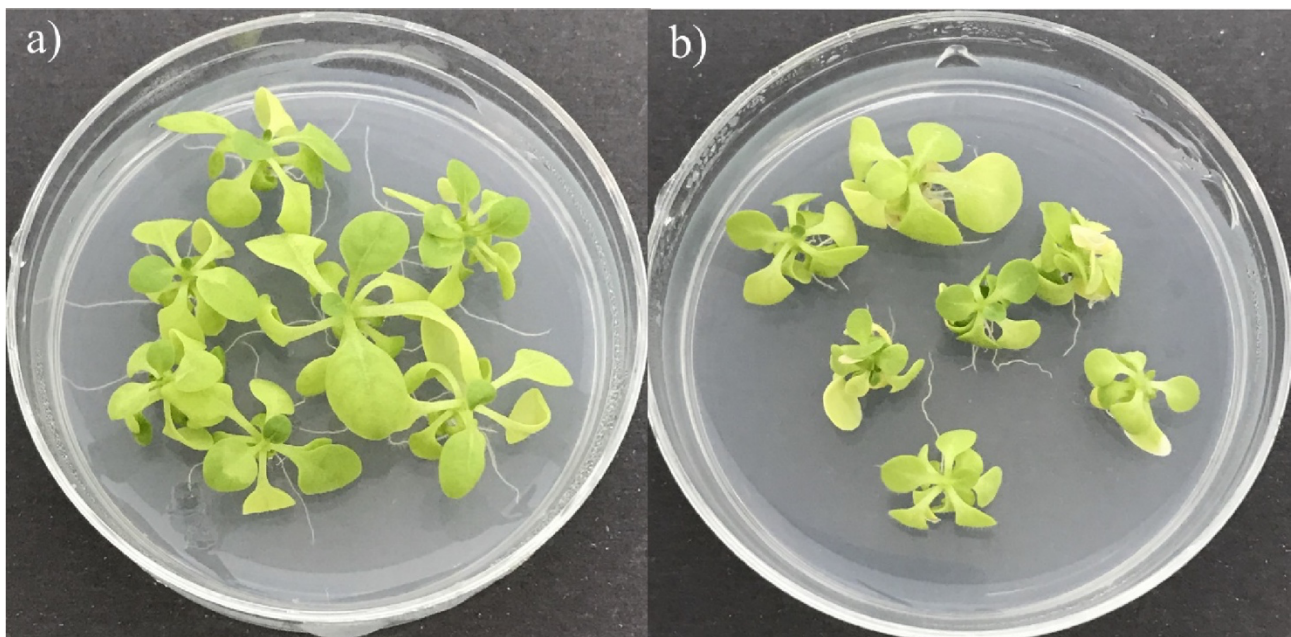

Figure 1. Effects of chaetocin on post-germination growth of *N. tabacum* seedlings at the 30th day of culture on MS medium. a) Control b) 5 μM Ch-treatment.
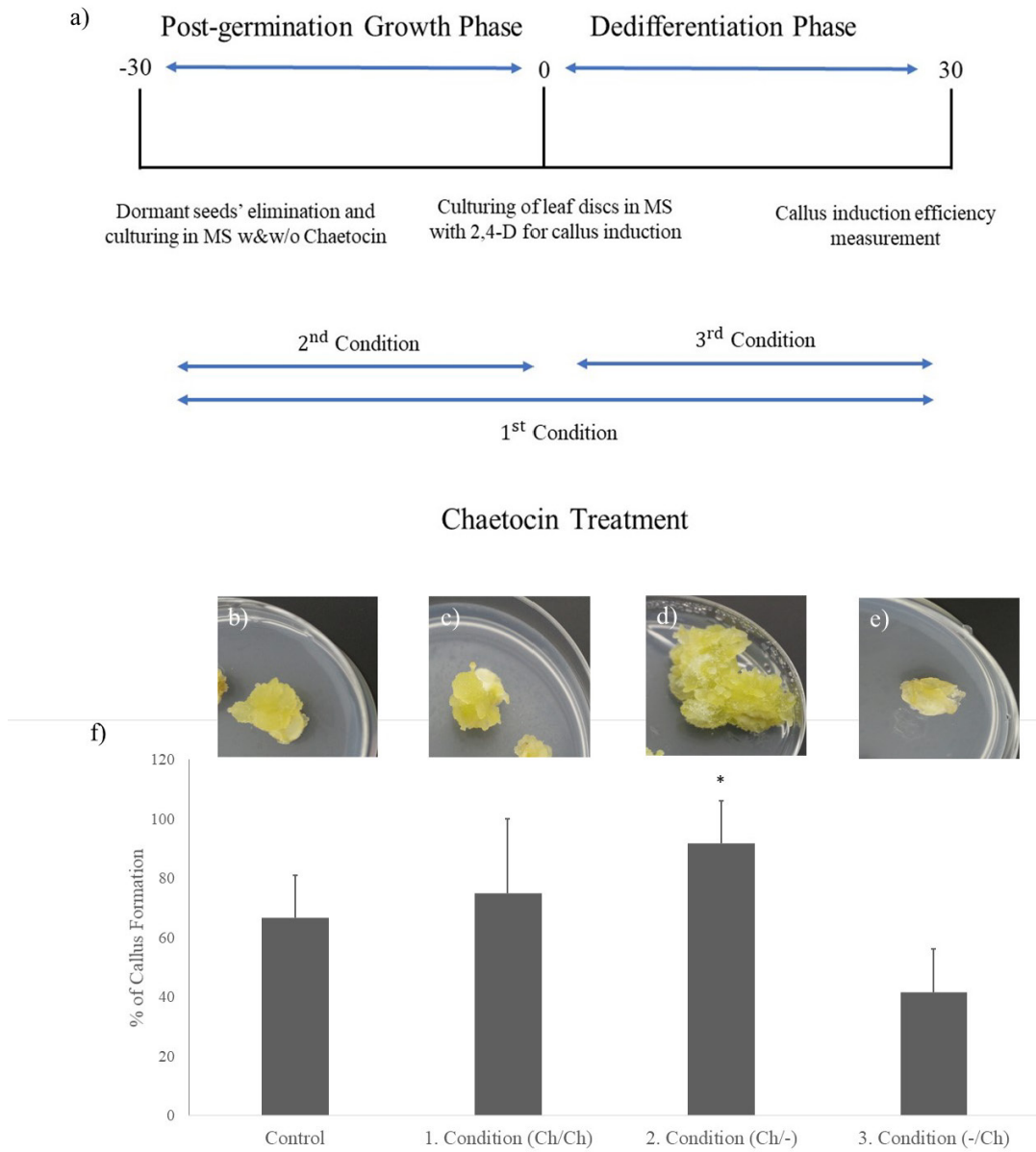
Figure 3. Time course treatment of Ch on *N. tabacum*. a) Schematic display of the time course treatment of Ch on *N. tabacum*, and plate photos for b) control, c) 1st condition, d) 2nd condition, e) 3rd condition, f) % of callus formation and Ch-treatment. There are around 1.1, 1.4- and 0.6-fold change increase in callus induction against control. Error bars = standard deviation. * p-value ≤ 0.05.
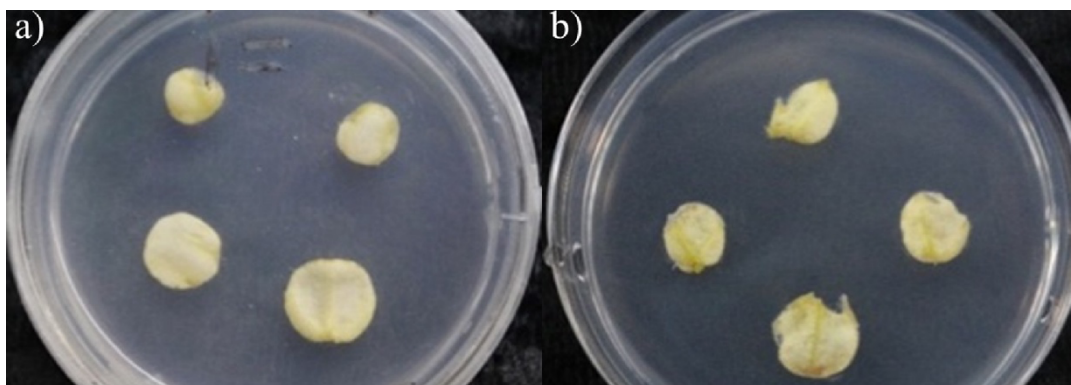

Figure 4. Leaf disc images at the 30th day of culturing of the experimental group in which 5 μM Ch was applied instead of 2,4-D during post-germination growth and callus formation a) control (MS medium), and b) 5 μM Ch treatment.

According to the results of qRT-PCR, all the target genes related to leaf differentiation, which are *AS1, KAN1, YAB3,* and *FIL*, were downregulated in the Ch-treatment group by 69, 45, 46, and 68%, respectively, compared to the control group (Figure 5).

4. Discussion
The principal characteristic of multicellularity is the cellular differentiation during development through specialized gene expression profile created by the major contribution of epigenetic regulators (Feng et al., 2010; Sugimoto et al., 2011). Among these regulators, histone-modifying enzymes (HMs) affect gene expression at the transcriptional level by remodeling the 3-D structure of chromatin (Zhou et al., 2020). Manipulating the activation of HMs enables the direction to the cell fate without changes at genetic compositions (Berenguer et al., 2017; Yu et al., 2017). In animal systems, several small-molecule compounds which target and inhibit HMs were identified and utilized for cancer, stem cell and development studies (Yu et al., 2017; Vaijayanthi et al., 2018; Verza et al., 2020). However, in plants, there are only a few studies that tested these chemical inhibitors based on HM homology between plants and animals (Li et al., 2014; Berenguer et al., 2017; Ruta et al., 2019). It is known that epigenetic regulators primarily associate with a wide range of cellular processes, including somatic embryogenesis, plant reprogramming and plant stem cell maintenance (Berr et al., 2011; Lee and Seo, 2018; Osorio-Montalvo et al., 2018; Singh et al., 2020). Therefore, characterizing new plant epigenetic inhibitors and elucidating their effects and understanding the regulation of main cellular mechanisms may help researchers to develop new manageable approaches in plant science and biotechnology.

Chaetocin, a histone lysine methyltransferase inhibitor, draws attention because of its utilization in cellular reprogramming, epigenetic research and the treatment of several human cancers (Greiner et al., 2005; Lai et al., 2015; Jeong et al., 2020). In this study, we investigated the presumption of Ch for being a new potential plant epigenetic modulator and analyzed the effects in growth and dedifferentiation processes in tobacco tissue culture for the first time.

We determined the appropriate time of Ch treatment (2. Condition) for the eligible callus induction rate, which was stated in the course from germination to callus formation. It is critical to determine optimal conditions for Ch treatment since this agent can create varying effects depending on the culturing cell type and the aim of the study, based on the time and dosage (Cherblanc et al., 2013; Jung et al., 2016).

When Ch applied to the tobacco seedlings, defective leaf formation observed (Figure 2), and, after the treatment of Ch during the post-germination growth phase, the expression of *AS1, KAN1, YAB3,* and *FIL* genes decreased by 69, 45, 46, and 68%, respectively (Figure 5), which have a critical role in building leaf polarity through inactivation of *KNOX* expression (Bowman and Eshed, 2000; Palermo and Dornelas, 2020). According to the previous findings on Ch, it was shown that this compound inhibits SUV39H1 and G9a HKMTs in animal systems (Greiner et al., 2005). These methyltransferases share conserved SET domains with plant SUVR4 HKMT (Thorstensen et al., 2006). Based on the evidence of defective leaf morphologies we observed, it can be assumed that Ch reduced H3K9me2/3 marks by inhibiting SUVH and SUVR group enzymes, which have crucial functions on the cellular differentiation and the maintenance of the cell identity in plant development (Thorstensen et al., 2006; Veiseth et al., 2011). In this manner, attenuated repressive chromatin marks lead to decreased expression of leaf identity genes compared to control due to the incomplete assignment of the cell fate. Therefore, the application of Ch at the post-germination growth phase of seedlings increased the callus formation 1.4 times more than the control (Figure 3d and 3f). These outcomes may have been the result of that chaetocin negatively affects the activity of repressive HKMTs, probably SUVH and SUVR enzymes, which are crucial for controlling gene expression in tobacco seedlings as in animal systems (Greiner et al., 2005; Thorstensen et al., 2006; Veiseth et al., 2011). The elimination of this epigenetic barrier may have triggered the molecular pathway, resulting in decreased expression of leaf identity genes.
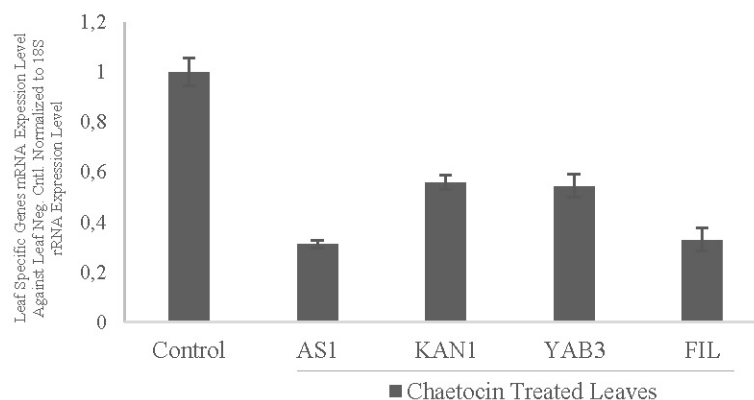

Figure 5. The effects of Ch treatment on the expression of major leaf polarity genes in the post-germination growth phase of *N. tabacum* (N:3, Error bars = standard deviation).

The inactivation of these leaf-polarity genes causes ectopic expression of some SAM genes in the leaf; those represent a less-differentiated cell stage and the facilitation for the entrance into cellular reprogramming, which is the basis of callus formation.

As the data obtained show, Ch affects cell fate transition and can be a potential epigenetic inhibitor in plants. This present work is a pioneer, exhibiting valuable data for detailed studies to understand the molecular mechanisms underlying the effects of chaetocin and the unknown properties of HM. Furthermore, the utilization of this agent needs high throughput methods like ChIP Quantitative PCR (ChIP-qPCR), genomic-based techniques, and cell-type-specific analysis. Hence, novel approaches can be developed at the tissue culture level to elucidate of epigenetic mechanisms and cellular differentiation/dedifferentiation processes in plant systems.

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