AN EVALUATION OF THE IMPACT OF DEMETHYLATING AGENTS TREATMENT USING TGS 16C *NICOTIANA BENTHAMIANA* REPORTER LINE

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Abstract: Epigenetics is one of the fastest-growing areas of science and has now become a central issue in biological studies of development, stress impact and disease. DNA methylation provides a way how to alter the gene expression pattern without disrupting or modifying the genome. Here, we demonstrate the potential of a *Nicotiana benthamiana* TGS 16C reporter line in the signalization of demethylating events caused by the activity of the so-called demethylating agents. These compounds have the ability to block or to interfere with the activity of methyltransferases, enzymes responsible for maintaining methylation marks on the replicating sequences. This *N. benthamiana* line carries a green fluorescent protein gene (GFP), whose promoter had been methylated and is thus inactive. By treating such plants with compounds with demethylating properties, their demethylating potential can be estimated by the effect on the re-established GFP expression in plant tissues.

Key words: DNA methylation, GFP, laser scanning, UV light, tissue cultures

INTRODUCTION

Epigenetic changes represent a brand for any change in genetic information that is not caused by mutation. The actual meaning of the sequence is not changed; epigenetic regulation change the way in which the cell express the genes that are already a part of its genetic code. These modifications do not change the DNA sequence, but instead, they affect how cells "read" genes. If the change is heritable, it is thus called „epimutation“ (Oey, Whitelaw 2014).

There are several types of epigenetic modifications, but these non-genetic alterations are tightly associated by two major epigenetic modifications: chemical modifications to the cytosine residues of DNA and histone proteins associated with DNA. A lot of these processes are proven or believed (Matzke, Mosher 2014) to be directed by small RNA, particles of the size of 21-26 base pairs. Depending on their origin, they often associate with specific proteins to create a nucleoprotein unit effectively inhibiting or interfering with the sequence identical or similar to its guide RNA strand (Finnegan, Matzke 2003).

Other mechanism inducing epigenetic changes is DNA methylation. When the sequence of a gene is methylated, it means that the so-called fifth base is present in the code. Aside of the guanine, thymine, cytosine and adenine, there is also methylcytosine (5-mC), a cytosine with a methyl moiety. When this moiety is attached to the cytosine included in the gene regulatory region, the sequence is often not expressed. Demethylating agents (DMTs) are compounds with ability to block the methylation of the DNA. By this, the previously not expressed sequences will lose its methylation, 5-mC will turn back to C.

There are various pathways in which the methylated sequence can become demethylated. The most commonly used DMT compounds are cytidine analogues such as 5-azacytidine (AC) and zebularine (ZEB). Once incorporated, the analogues covalently trap the DNA methyltransferases and mediate their degradation, leading to a passive loss in DNA methylation in the cell (Stresemann, Lyko 2008, Yoo et al. 2004). Another way how to introduce demethylating changes into the DNA is

using 5,6-dihydro-5-azacytidine (DHAC), which is a hydrolytically stable congener of 5-azacytidine (Matoušová et al. 2011). This compound blocks the methylating pathway by sealing off the bonding between methyltransferase and the methyl group donor.

Epigenetic changes comprise of diverse pathway machinery, turning the genes expression on and off according to the organism's current needs. Epigenetic circuitry seems to be very dynamic – different genes are needed in different developmental, climatic or otherwise stressful conditions (Chinnusamy, Zhu 2009, Turck, Coupland 2013, Walter et al. 2013).

To investigate the activity of the DMTs, a transgenic line of tobacco was used. *Nicotiana benthamiana* is a well-established model plant in molecular biology. The TGS 16S *N. benthamiana* line used in this study has originated from the 16C line (Jones et al. 2001). These plants carry the green fluorescent protein gene (GFP), whose expression has been repressed by RNA dependent DNA methylation caused by the infection of virus carrying a part of the GFP sequence promoter. This had led to the gene becoming silenced and also being heritable independently on its RNA initiator (Law, Jacobsen 2010). This particular trait can serve as an ideal marker of successful demethylation, when by treating such seed with compound having demethylating effect, the result should be clearly visible under the UV light. If the GFP is expressed, the plant will appear as green under the UV. If there is no active expression, the plants will appear as red.

This study's aim was to monitor the effect of DMTs on the expression of the green fluorescent protein. The purpose of this was to determine whether the TGS 16C line could be used as an indicator of a demethylating activity of any putative demethylating compound. To measure the effect, three types of DMTs were used, utilizing two different pathways of DNA demethylation.

MATERIALS AND METHODS

Ten batches of solid ½ MS media (Murashige, Skoog 1962) were prepared. The DMTs used were 5-azacytidine, zebularine and 5,6-dihydro-5-azacytidine (Table 1). For each of these compounds, three ½ MS media solutions were made, each with different concentration of 20 µM, 40 µM and 80 µM, thus resulting in 9 variants. Each of this variant had been prepared in two replications. The untreated TGS 16C seeds were used as a control variant, sown onto the ½ MS media free of additives.

Table 1 List of used DMTs containing the code of the compound, molar mass and structural formula

5-Azacytidine (AC)	**Zebularine (ZEB)**	**5,6-Dihydro-5-azacytidine acetate salt (DHAC)**
244.21 M	228.20 M	306.27 M

The seeds were sterilized in 96% ethanol, washed in distilled water multiple times and sown onto the Petri dish containing 20 ml of solid ½ MS media enriched with the respective DMTs. There were 20 seeds per each plate.

After 14 days, the seedlings from one Petri dish for each variant were transferred into pots filled with soil and placed in greenhouse. The seedlings from the second replication of each variant were left to grow on the enriched media for another 7 days (21 days on media in total).

After 20 days in soil, the first batch of plants was subjected to analysis using UV hand lamp and Molecular Imager Pharos FX™ Plus Systems. Images were made by combining the separate images taken using CY3 and FITC channels on medium setting and merging them into one. As a standard of GFP expression, *N. benthamiana* 16C line with non-methylated promoter was used, together with wild type *N. benthamiana* (WT) as negative control.

After additional 5-6 weeks (8-9 weeks in total in soil) growing in the greenhouse, analysis of GFP expression in mature leaves was performed.

RESULTS AND DISCUSSION

Germinability of the seeds

As can be seen from Table 2, the addition of the DMTs into the growth media had pronounced effect on the germinability of the seeds. The lowest germinating capacity in total had the seeds treated with DHAC, resulting in germinability as low as approx. 46%. In the untreated variant 90% of the seeds were viable using the same growth conditions with the exception of the DMTs addition.

Table 2 Seed germinability

Code of the compound	Concentration in media (μM)	Germinability (1. replication)	Germinability (2. replication)	Germinating capacity in total
ZEB	20	11/20	14/20	62.5%
	40	16/20	17/20	82.5%
	80	13/20	13/20	65%
AC	20	12/20	15/20	67.5%
	40	10/20	10/20	50%
	80	16/20	16/20	80%
DHAC	20	10/20	10/20	50%
	40	8/20	8/20	40%
	80	12/20	8/20	50%
Untreated Cont.	-	17/20	19/20	90%

Seedlings grown on the media with DMT had shown severe growth retardation correlated with the DMT concentration in media with the exception of DHAC, whose growth rate was comparable to untreated control (Figure 1).

Figure 1 Seedling growth rate comparison - top row from left to right: Untreated control, ZEB 80 µM; bottom row from left to right DHAC 80 µM and AC 80 µM.

Effect on growth

After germination, the growth rates of AC and ZEB treated seedlings had dwindled and continued to do so while being under the influence of the DMTs. After being removed from the media, the plantlets had slowly regained its vitality to a point, when their growth rate was almost identical with the untreated control. This applies both for the 14 days and 21 days treatment variants. Aside from the very low germinability levels, DHAC had the smallest effect on the overall growth. One of the potential reasons for this decline in growth can be the demethylation of the otherwise epigenetically inactivated transposable elements (reviewed in Slotkin, Martienssen 2007) and their remethylation after the demethylating stimulus was removed.

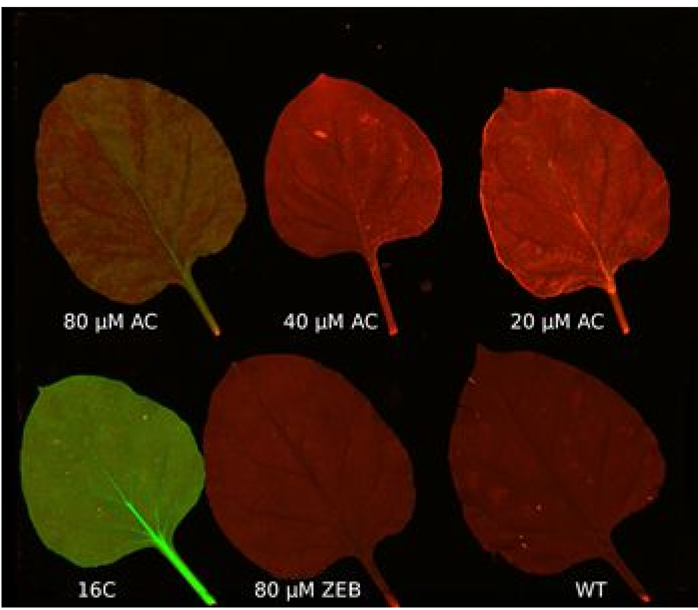
GFP signal detection

In the first stages of the experiment, the plants were scanned with the UV handlamp, which provided preliminary results of the GFP expression. Among the plantlets grown on soil for 20 days, the results
of this scanning suggested active GFP expression in cotyledons and young true leaves (Figure 2). However the images obtained using the handlamp were too blurry to provide any definite evidence. Some of the seedlings were thus subjected to laser scanning, which ultimately led to destroying of the scanned plantlets. As can be seen in Figure 2, some of the 80 µM AC and 80 µM ZEB treated ones displayed positive positive signal of expression of GFP. Neverthenless when mature leaves of plants under the same treatment were scanned later, there was not observed any GFP signal with the exception of 80 µM AC variant, where the leaf venation seemed slightly more green (Figure 3). It is thus possible that the occurrence of the expressed GFP protein in DMTs treated plants was so rare, that the plants chosen in the first round were the only ones expressing it. Other explanation for the low rate of GFP signal detection in true leaves is the reversion of the promoter methylation status back to the original state.

In true leaves, some demethylating activity was detected only in the case of 80 µM AC treated plants (Figure 3). No demethylating activity was detected in the plants treated with DHAC, which in all stages of development remained red when subjected to scanning. Similarly, zebularine treated plants had no GFP activity in mature leaves, although the initial results suggested that there had been active demethylation in some cases during the first true leaves stage. All the pictures shown represent the results of at least five separate scannings. All of the leaves subjected to scanning were carefully chosen to avoid misinterpretation of the GFP signal due to leaf damage.

Figure 2 GFP expression in cotyledons and young true leaves of N. benthamiana

Figure 3 N. benthamiana true leaves scanning



CONCLUSION

Application of the DMTs caused overall loss of seed germinability and severe retardation of growth in the first stage of experiment, although when removed from the DMTs influence, the plants had slowly regained its vitality and their growth rate became comparable with untreated control. The demethylation
of the GFP promoter was observed mainly during the cotyledon/young true leaves stage, while the plants were still under the direct influence of the DMTs or relatively freshly removed from it into the soil. In the stage of true leaves, the 80 µM AC had been the only variant with detectable GFP expression.

ACKNOWLEDGEMENT

This work was supported by IGA ZF MENDELU 2015. Authors are grateful to dr. Michael Wassenegger and dr. Athanasios Dalakouras for provided support. AC and DHAC used in this study were kindly provided by dr. Miroslav Otmar and dr. Marcela Krečmerová from Institute of Organic Chemistry and Biochemistry AS CR, v.v.i.

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