EFFECT OF 5-AZACYTIDINE ON CALLUS INDUCTION AND PLANT REGENERATION POTENTIAL IN ANTHER CULTURE OF WHEAT (*TRITICUM AESTIVUM* L.)

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Summary. Generation of double haploid wheat plants *via* anther culture is an important biotechnological method, which permits significant shortening of the breeding process. The response in anther culture is highly genotype-specific, so it is necessary to find ways for its modification. One such approach could be based on changes of DNA methylation pattern. In the present study we tested the effect of 5-azacytidine (5-azaC), a compound causing extensive hypomethylation of genomic DNA, on wheat response in anther culture. We observed an increase of callus induction (up to 38%) and plant regeneration (up to 50%) at particular concentrations within the picomolar and nanomolar range. On the other hand, the effect of 5-azaC proved to be not straightforward and resulted in a complicated response pattern.

Keywords: wheat, DNA methylation, 5-azacytidine, anther culture, regeneration.

Abbreviations: 5-azaC – 5-azacytidine; cv. – cultivar; PAR – photosynthetically active radiation; MTase - methyltransferase;

INTRODUCTION

The generation of fertile double haploid plants is among the most practical tools of wheat biotechnology, which permits significant shortening of the breeding process (Hu, 1997). Two main approaches are currently in use - anther culture (Tuvesson et

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al., 2000) and microspore culture (Gustafson et al., 1995). Despite its limitations anther culture is the more practical one since it is not as laborious and time-consuming as microspore culture. On the other hand, anther culture has one fundamental drawback with great practical importance - the highly genotype-specific response (Niemirowicz-Szczytt, 1997). Thus, finding approaches to overcome this strong genotype specificity could permit wider application of this method on commercially important varieties with low or no response in anther culture.

Gene expression patterns are easily influenced by changing DNA methylation pattern because methylation of cytosine residues in DNA provides a mechanism for gene control either *in vivo* or *in vitro* (Finnegan et al., 1998, 2000; Brown, 1989). The regulatory effects of DNA methylation could be divided in two broad categories: (i) specific, when particular gene activity is directly influenced by methylation of its regulatory regions (Habu et al., 2001) and (ii) general, when methylation causes changes in the chromatin structure (Nakao, 2001; Juttermann et al., 1994). Tsaftaris and Polidoros (2000) have recently discussed the importance of DNA methylation and its effects on plant breeding.

In this work we tested the hypothesis that decreasing of DNA methylation levels could unlock the callus induction potential in anther culture, thus making this technique genotype-independent. For that purpose we used 5-azacytidine (5-azaC) known as a potent inhibitor of DNA methylation (Jones, 1985).

MATERIALS AND METHODS

Four winter wheat (*Triticum aestivum* L.) cultivars were used in this study. Cv. Svilena showed an extremely high anther culture response, while cv. Dobrotitsa was almost non-responsive as observed in previous experiments (Belchev et al., 2000). The other two cultivars, Aglika and Enola showed moderate and low androgenic responses, respectively.

The seeds for anther-donor plants were germinated and vernalized at 4°C for 45 days, then the temperature was increased up to 15°C(day)/10°C(night) for 30 days and after that plants were grown at 20°C (day)/15°C(night). The spikes with anthers containing microspores at mid- to late uninucleated stage (He and Onyang, 1984) were collected and stored at 4°C for 7 days. The induction medium was C17 (Wang and Chen, 1986) in which sucrose was replaced with maltose and filter-sterilized 5-azacytidine was added at the following concentrations: 1, 5, 10, 50, 100, 500 pM; 1, 5, 10, 50, 100, 500 nM, 1, 5, 10, 50, 100, 500 and 1000 μM. Anthers from 5 spikes were excised and plated in 5 test tubes containing 10 mL induction medium. There were 5 replications with 150 anthers (5 tubes by 30 anthers) for each 5-azaC concentration tested. Anthers were incubated at 28°C in darkness for 30 days.

Emerging calli and embryos were transferred to regeneration medium 190-2

(Zhuang and Jia 1983) and grown at 25°C, 18.8 mmol.m^{-2}.s^{-1} PAR, 16h day/8h night till whole plants were regenerated. Two androgenic traits were estimated: i) callus induction - measured as the number of calli induced per 100 anthers cultivated and ii) plant regeneration – measured as a percentage of green and albino plants regenerated from calli transferred to the regeneration medium;

Results and discussion

5-azaC causes hypomethylation of DNA by inhibiting the methyltransferase (MTase, E.C. 2.1.1.37) activity when included into DNA as a substitute to deoxycytosine (Juttermann et al., 1994). The compound is included in DNA mainly during replication and, to a lesser extent, during reparation processes (Brown, 1989; Habu et al., 2001).

In our experiments 5-azaC had no effect on the callus induction in the anthers of non-responding and low-responding varieties (cv. Dobrotitsa and Enola, respectively), even at the lowest concentrations used. Taking into account the compound mode of action, this result suggested that there were no active DNA replication/reparation processes in the anthers of these cultivars when subjected to *in vitro* cultivation in the presence of auxin analogs. Thus, it could be speculated that "low-responsive" phenotype could be associated either with the response to auxins in the culture media or with the intracellular hormonal balance. In our future work we will focus our attention especially on analyzing the differences in auxin perception in responsive and non-responsive varieties.

In the highly-responding and moderate-responding varieties (cv. Svilena and Aglika, respectively) 5-azaC caused a clear increase in both haploid callus induction and plant regeneration at several concentrations which were below the micromolar range used by other investigators. Despite the lack of more detailed information at this stage, such an increase demonstrates that the genes responsible for callus induction show sensitivity to DNA methylation levels.

The toxic effect of 5-azaC increased with increasing its concentration and no callus induction or plant regeneration was observed at concentrations above 1.10^{-4} M.

The effect of 5-azaC on callus induction differed between the two varieties (Fig. 1). In cv. Svilena an increase of up to 38% was observed at 1.10^{-9} M as well as of up to 25% in the range $1 – 5.10^{-11}$ M while no increase in cv. Aglika was observed at all.

During the course of our work we have encountered an unexpected 'oscillatory effect' of 5-azaC on both parameters measured. This effect was more pronounced on callus induction (Fig. 1) where four main peaks might be identified. It is known that 5-azaC has a toxic effect on cells caused mainly by sequestering MTase (Juttermann et al., 1994). This could explain its toxicity within the upper concentration range (above 1.10^{-4} M), however the reason for the 'oscillatory' pattern as well as the decreased callus induction observed at concentrations below 1.10^{-9} M is not known. A

plausible explanation is that it is caused by minor changes of the methylation status of some yet unknown regulatory genes and the resulting disbalance between their antagonistic products. Thus, our results render cv. Svilena as an appropriate model system for analysis of the early events in plant regeneration.

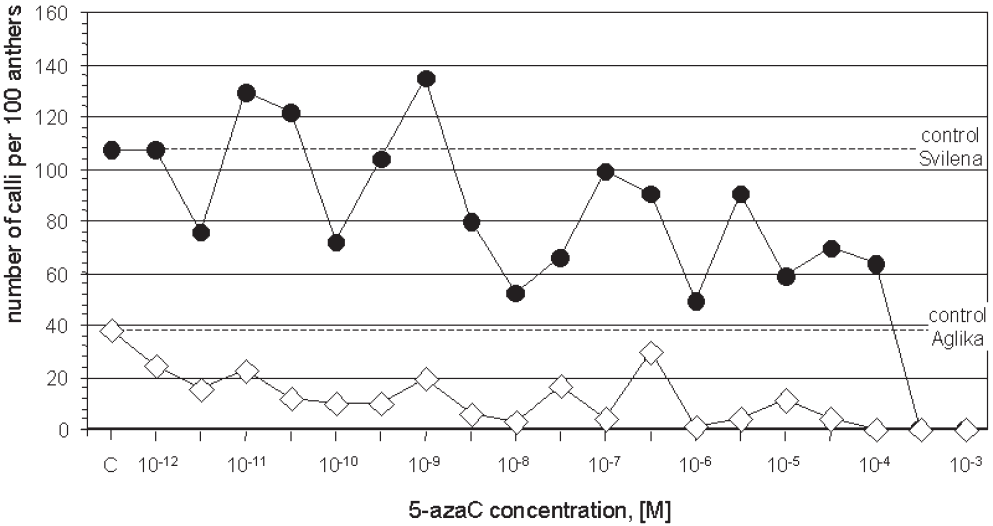

Figure 1. Effect of 5-azacytidine on callus induction. The effect was measured as the number of calli (embryoids) induced per 100 anthers cultivated. Data for cv. Svilena are shown with ●; for cv. Aglika with ◇. The relative controls for each cultivar are presented with punctuated lines. Unlabelled marks correspond to 5.10^{-n} concentrations of 5-azaC as described in Materials and Methods.

An important practical result from the present study is the possibility to increase the regeneration of fertile double haploid plants at particular 5-azaC concentrations (Fig. 2). In cv. Aglika plant regeneration was increased up to 35% in the range $5.10^{-10} – 1.10^{-8}$ M as well as at 1 - 5.10^{-11} M. The effect of 5-azaC on plant regeneration in cv. Svilena was not as pronounced as in cv. Aglika. A sharp increase of 27% was observed at 1.10^{-6} M.

What is more interesting, in both varieties a sharp increase of some 22% was observed at the highest sub-lethal 5-azaC concentrations. This increase in plant regeneration is not matched by an increase in callus induction. The 'oscillatory' pattern in plant regeneration was also observed but it was more pronounced in cv. Aglika as compared to cv. Svilena.

5-azaC had an opposite effect on the analyzed parameters in both cultivars. In the highly responsive cv. Svilena 5-azaC increased callus induction but had a weak effect on plant regeneration. In the moderate responsive cv. Aglika 5-azaC caused a sharp increase in plant regeneration but had no effect on callus induction.

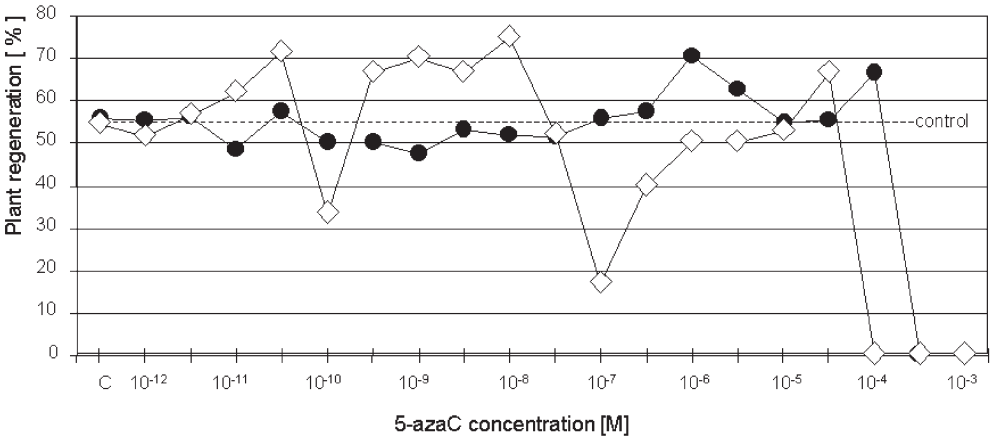

Figure 2. Effect of 5-azacytidine on plant regeneration. Plant regeneration was measured as percentage of all (green and albino) plants regenerated from calli transferred to the regeneration medium. Data for cv. Svilena are shown with ●; for cv. Aglika with ◇. Since the controls for each cultivar are almost similar a single punctuated line was used for consistency. Unlabelled marks correspond to 5.10^{-n} concentrations of 5-azaC as described in Materials and Methods.

In order to prove most of these speculative conclusions analysis of DNA methylation levels of anthers of both responding varieties at each 5-azaC concentration is under current investigation. Of particular interest was also the effect of 5-azaC treatment on seed storage protein expression. Preliminary results have demonstrated that some seed proteins show variations in their expression levels that might be related to 5-azaC treatment (data not shown).

Improvement of plant regeneration protocols is of great importance for both fundamental research and biotechnological applications. Our data along with the results from the analysis of other key parameters of the regeneration process (data not shown) indicate clearly that the use of 5-azacytidine to manipulate DNA methylation patterns has the potential to help in designing more efficient procedures for dihaploid plant regeneration.

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