Regular paper

Changes of DNA methylation and hydroxymethylation in plant protoplast cultures

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Cytosine methylation patterns in higher eukaryotes are important in gene regulation. Along with 5-methylcytosine (5-mC), a newly discovered constituent of mammalian DNA, 5-hydroxymethylcytosine (5-hmC), is the other modified base in higher organisms. In this study we detected 5-hmC in plant protoplast DNA and demonstrated its increasing content during the first 72 hrs. of protoplast cultivation. In contrast to 5-hmC, the amount of 5-mC decreased during protoplast cultivation. It was also found that 5-hmC did not primarily arise as a product of oxidative DNA damage following protoplast culture.

INTRODUCTION

The plant protoplast system represents one of the most spectacular examples of cell de-differentiation, which is characterized by large-scale chromatin rearrangement. The fully differentiated, non-dividing mesophyll cells, isolated from donor tissues by cell-wall degrading enzymes are able (upon the application of phytohormones) to proliferate, form calli, and regenerate into plants. During the cells' protoplastization, it is mostly whole heterochromatin (chromocenters) that is transiently de-condensed. Later, new chromocenters are assembled, and a new heterochromatin status is acquired at the time when the protoplast-derived cells re-enter the cell cycle (Ondrej et al., 2009).

Chromatin (the complex of DNA, proteins, and RNA) represents the substrate for most processes in the cell nucleus which involve replication, transcription, as well the maintenance of genome stability and integrity. Moreover, chromatin must retain the ability to make genetic information accessible when needed. This regulation involves levels of chromatin condensation that are connected with epigenetic markers such as DNA methylation and histone modifications (Exner & Hennig, 2008). The methylation of cytosines (5-mC) in DNA has been found in most eukaryotes, including plants, animals, and fungi. Additionally, it is generally accepted that DNA methylation plays an important role in gene repression. It is now evident that epigenetic modifications, including DNA methylation, regulate heterochromatin level and control, as well as the processes of cell differentiation (Avramova, 2002). It is likely that a cell must establish a new balance between the euchromatin and heterochromatin in order to change its differentiation state, since actively transcribed genes are located within open euchromatin; the silenced, repressed genes are located in the heterochromatin. Terminal cell differentiation involves large-scale chromatin remodelling, which turns most of the open euchromatin into condensed heterochromatin (Grigoryev et al., 2006).

DNA might not be modified by way of cytosine methylation alone. Recent studies have shown a high level of 5-hydroxymethylcytosine (5-hmC) in mammalian embryonic stem cell DNA, as well as in Purkinje cells and the brain (Kriaucionis & Heintz, 2009). Conversion of 5-mC to 5-hmC is mediated by enzymes of the TET family (Tahiliani et al., 2009) or by oxidative damage (Valinluck et al., 2004). Additionally, 5-hmC significantly inhibits the affinity of the methyl-CpG-binding domain of the MeCP2 protein, which plays a key role in methylation-mediated gene silencing (Valinluck et al., 2004).

We focused on the detection of 5-hmC in plant DNA, and compared global epigenetic changes in DNA methylation and hydroxymethylation during the first 72 hrs. of plant protoplast cultures in two different plant species, *Cucumis sativus* and *Brassica oleracea*. The cucumber protoplast cultures were only able to proliferate into calli; in contrast, the *B. oleracea* protoplast cultureswere able to regenerate into plantlets. The levels of DNA modifications were also compared with both the protoplast viability and ROS production modulated by ascorbic acid, to determine if the 5-hmC originated *via* the oxidative damage of 5-mC.

MATERIALS AND METHODS

Protoplast isolation and culture. Protoplasts were isolated from leaves of growing young plants *in vitro* by standard protocols — *B. oleracea,* var. *botrytis* cv. SIRIA according to Navrátilová et al., (1997); and *C. sativus* cv. MARKETER according to Gajdová et al., (2007). The protoplasts of *B. oleracea* were cultured in B medium (Pelletier et al., 1983), and those of *C. sativus* in LCM1 medium (Gajdová et al., 2007).

For the DNA isolation, 5 ml of protoplast suspension at a cell density of 1.5×10^5 per ml was cultured in tissue culture flasks (50 ml volume). For other measurements, 1.5 ml of protoplast suspension at the same density was cultured in Petri dishes (35 mm). Ascorbic acid was added to the cultures immediately after protoplast isolation (PI) at two concentrations: 50 and 100 mg/l of culture media. The protoplasts were cultured in an incubator at 25°C in the dark.

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Abbreviations: 5-mC, 5-methylcytosine; 5-hmC, 5-hydroxymethylcytosine; PI, protoplast isolation; ROS, reactive oxygen species.
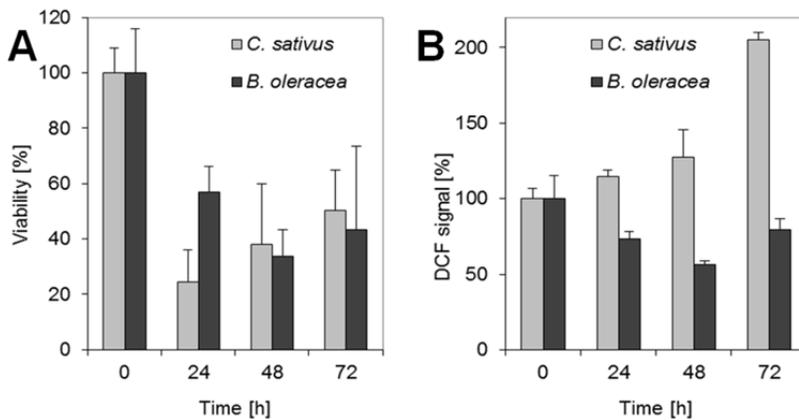
Figure 1. Measurements of viability and ROS production in protoplast cultures during culture period.
Subset A shows dramatic decreasing of viability of protoplasts after their isolation and slight growth of the viability at the end of the culture period for both studied species — *C. sativus* and *B. oleracea*. **Subset B** showed increasing of ROS production in *C. sativus* protoplast cultures during culture period (72 h). In contrast to *C. sativus*, protoplasts of *B. oleracea* are able to suppress ROS production.

Determination of cell viability by FDA method. Fluorimetric determination of the vitality, using FDA (fluorescein diacetate), was carried out in microplates using a microplate reader (Synergy HT, Biotek, USA). FDA (5 µl, 25 µg/ml) was added to 100 µl of cell suspension, and after 10 min of incubation the fluorescence intensity was measured (λ_{exc} — 485 nm and λ_{em} — 515 nm).

ROS measurement. The concentration of H_2O_2 in protoplast suspension was determined fluorimetrically using the fluorescent probe 2,7-dichlorodihydrofluorescein diacetate (H_2DCF DA). H_2DCF DA can permeate through the cell membrane into the cell, where it is metabolised by cell esterases into H_2DCF. Next, the H_2DCF is oxidized by reactive oxygen species into fluorescent DCF. The H_2O_2 assessments were performed on a microplate reader (Synergy HT, Biotek, USA). Five microliters of 0.2 mM H_2DCF DA fluorescent probe was added into 100 µl of cell suspension. The fluorescent signal (λ_{exc} — 485 nm and λ_{em} — 515 nm) was measured immediately after the addition of the probe, as well as after incubation of the cell suspension for 1 hour at 26°C in the dark. For each experimental variant, three measurements were conducted for each of the three independent sets of samples (i.e., nine values were obtained). The data are presented as the mean + standard deviation.

DNA methylation and hydroxymethylation level measurement. DNA was isolated from the protoplast cultures at 0, 24, 48 and 72 hrs after protoplast isolation (PI) and from donor leaves by standard CTAB procedure. DNA modifications were quantified using colorimetric assays with MethylFlash Hydroxymethylated and Methylated DNA Quantification Kits (Epigentec, USA) according to the manufacturer's protocols. The relative quantities of 5-hmC and 5-mC were calculated according to manufacturer's formulas using the manufacturer's standards.

RESULTS AND DISCUSSION

Two protoplast culture systems were used to study global epigenetic changes of DNA. The first one, based on *B. oleracea*, is a highly regenerative system that gives a stable high number of regenerated plantlets, which have been used in somatic hybridization (Navrátilová *et al.*, 1997). In the second system, *C. sativus*, the protoplasts are able to form calli or somatic proembryos; however, no plants have been obtained by this means of culture, no matter how many genotypes and media modifications were used (Gajdová *et al.*, 2007; Ondrej *et al.*, 2009a).

The two protoplast culture systems did not significantly differ in the viability of the protoplasts during the first 72 h of culture (Fig. 1a), but they did differ in their ROS production (Fig. 1b). Using a ROS-specific probe, an increasing level and higher amounts of ROS were observed during the culture period of *C. sativus* protoplasts compared to *B. oleracea*, which showed a decreasing tendency after protoplast isolation. At 72 h after PI, the ROS level in the *B. oleracea* protoplasts increased, which is connected with cell division. An addition of ascorbic acid at a concentration of 50 mg/l to the culture media strongly inhibited ROS production in the *B. oleracea* and only moderately and transiently in *C. sativus* (Fig. 2). Ascorbic acid at 100 mg/l immediately decreased the level of ROS to below the detection level in both plant species. The ROS reduction effect of ascorbic acid in the cucumber protoplast culture was also described in Ondrej *et al.* (2010).

At the same time points at which the ROS level and viability were measured, the amounts of 5-hmC and 5-mC were determined. The amount of 5-mC in the DNA of freshly isolated protoplasts of both species was nearly the same (about 4% of relative cytosine content), and decreased during the culturing of both types of protoplasts (Fig. 3a, b). At 72 hrs after PI, the content of 5-mC was significantly lower in the cucumber DNA (Fig. 3a) than in the DNA of the *B. oleracea* protoplasts
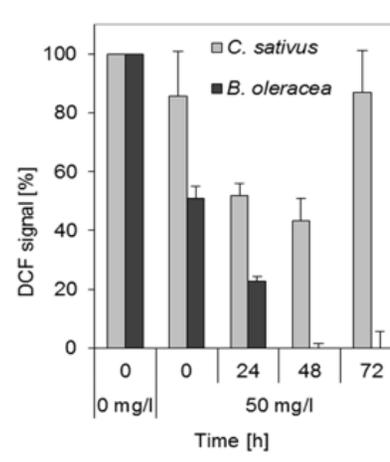
Figure 2. The effect of ascorbic acid addition to culture media on ROS production.
An addition of ascorbic acid at a concentration of 50 mg/l to the protoplast culture media strongly reduced ROS levels in the *B. oleracea* and only moderately and transiently in *C. sativus* protoplasts.
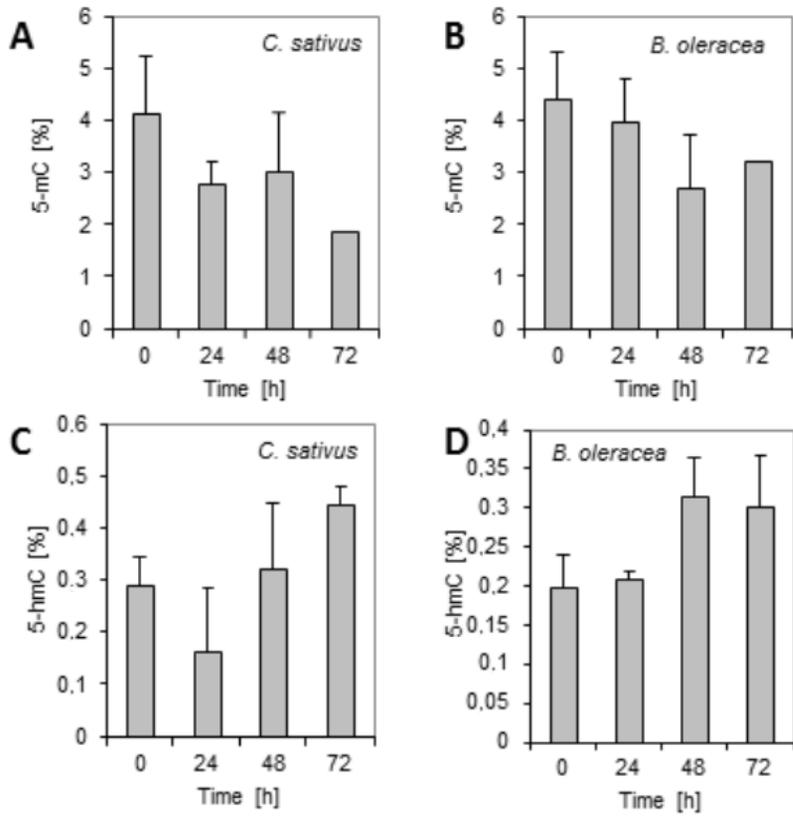
Figure 3. The global changes of 5-mC and 5-hmC content in DNA of protoplast cultures during culturing.
The content of 5-mC (% of cytosines calculated from standards) in the DNA of freshly isolated protoplasts of both species was nearly the same and decreased during the culturing of both types of protoplasts **(A, B)**. In contrast to 5-mC, the content of 5-hmC has increasing tendency during culturing of *C. sativus* and *B. oleracea* protoplasts **(C, D)**.
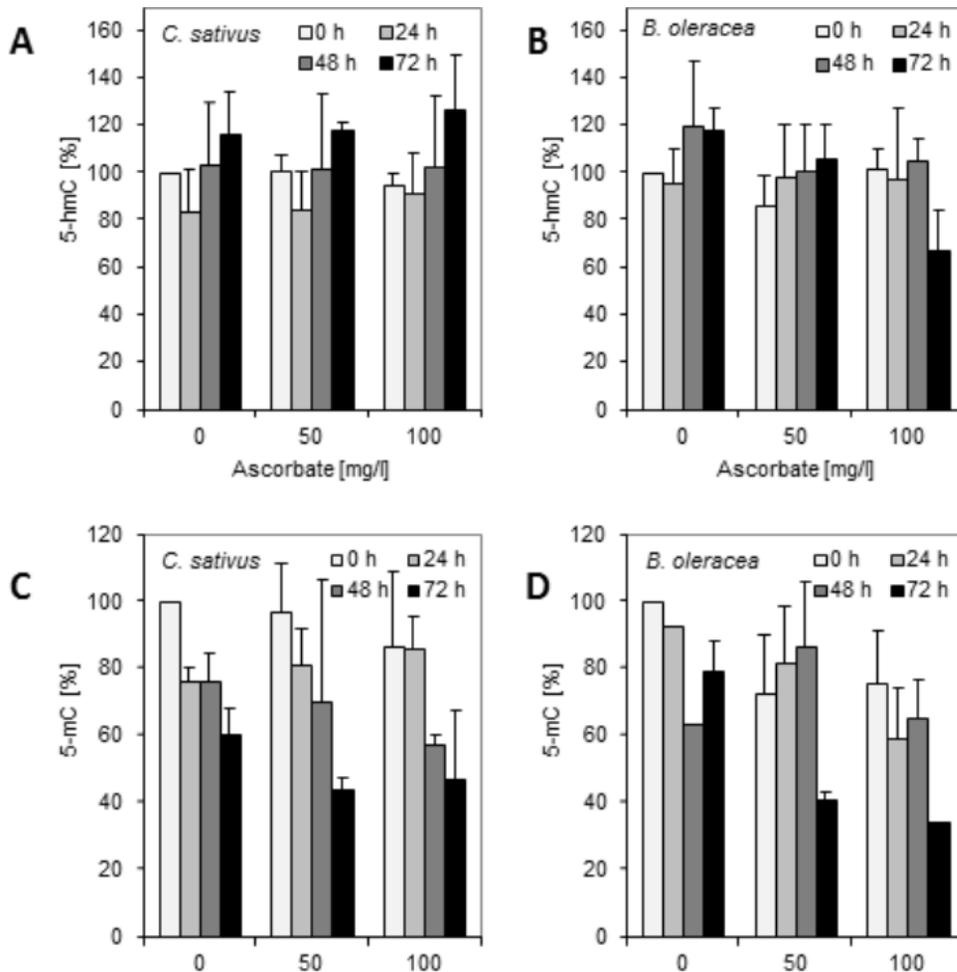
Figure 4. Changes of 5-hmC and 5-mC levels in *C. sativus* and *B. oleracea* protoplast cultures during culturing in the presence of ascorbate.
In comparison to untreated protoplast with ascorbic acid, the treated protoplasts of *C. sativus* did not show any significant changes in 5-hmC levels **(A)**. But ascorbic acid treated protoplasts of *B. oleracea* displayed a decreasing tendency in 5-hmC level at 72 h after PI **(B)**. The 5-mC levels in the protoplast cultures of both studied species treated with ascorbic acid showed a decreasing tendency comparing to untreated cultures **(C, D)**.

(Fig. 3b). Additionally, the methylation level of DNA in freshly isolated protoplasts did not differ from the methylation levels in leaves that were used for protoplast preparation.

In contrast to 5-mC the amount of 5-hmC displayed an increasing tendency with culture duration in both protoplast systems; and 72 h PI, the amount of 5-hmC in cucumber DNA was significantly higher (Fig. 3c) than in the DNA of the *B. oleracea* protoplasts (Fig. 3d). In the human, Nestor *et al.* (2012) found that the global 5-hmC content of normal tissues is highly variable, does not correlate with the global 5-mC content, and decreases rapidly as cells from a normal tissue adapt to cell culture.

As mentioned above, the conversion of 5-mC to 5-hmC is mediated by enzymes from the TET family or by oxidative damage. It is generally accepted that ROS are responsible for DNA damage, and could be responsible for the recalcitrance of plant protoplasts (Papadakis *et al.*, 2002). However, the ROS levels in the cucumber protoplast cultures corresponded with the 5-hmC levels; conversely, the ROS levels in the *B. oleracea* protoplasts decreased during the culture period, but the 5-hmC level increased. We also showed that application of ascorbic acid did not produce significant global changes in DNA hydroxymethylation in the *C. sativus* cultures. In contrast, the levels of 5-hmC displayed a decreasing tendency in the *B. oleracea* protoplast cultures at 72 h after PI when treated with either concentration of ascorbic acid, and no ROS signal was detected (Fig. 4a, b). This shows that the detected changes in DNA hydroxymethylation are mainly independent of DNA damage and are probably due to enzymatic modifications with a small proportion contributed by ROS. The 5-mC level in the protoplast cultures treated with ascorbic acid showed a decreasing tendency comparing to untreated cultures (Fig. 4c, d).

In cucumber protoplasts, 48 and 72 hrs. after PI is the time when heterochromatin blocks (chromocenters) reassemble and the protoplast-derived cells re-enter the cell cycle (Ondrej *et al.*, 2009b; 2010). At those times, we measured the highest levels of 5-hmC and the lowest levels of DNA methylation. As mentioned above (Valinluck *et al.*, 2004), DNA hydroxymethylation decreases the binding affinity of proteins playing a role in heterochromatin formation. The present results reflect, and could be responsible for, the newly reformed and low density heterochromatin status in protoplast nuclei before their first division; additionally, they could also be responsible for the process of cell reprogramming. In humans, mutations in TET genes or their knocking down, which reduce the ability of cells to hydroxymethylate cytosine , lead to pathological differentiation of stem cells and to cancers (Wu & Zhang, 2011; Tsukada, 2012).

Acknowledgements

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