Biochemical and Biophysical Research Communications 447 (2014) 285–291

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

The age-dependent epigenetic and physiological changes in an *Arabidopsis* T87 cell suspension culture during long-term cultivation

Aleksandra Kwiatkowska [a,*], Jacek Zebrowski [b], Bernadetta Oklejewicz [c], Justyna Czarnik [a], Joanna Halibart-Puzio [b], Maciej Wnuk [c]

[a] Department of Botany, University of Rzeszow, Kolbuszowa, Poland
[b] Department of Plant Physiology, University of Rzeszow, Kolbuszowa, Poland
[c] Department of Genetics, University of Rzeszow, Kolbuszowa, Poland

ARTICLE INFO

Article history:
Received 24 March 2014
Available online 4 April 2014

Keywords:
Arabidopsis thaliana cell suspension culture T87
Aging
Epigenetics
Methylation
Photosystem II

ABSTRACT

Plant cell suspension cultures represent good model systems applicable for both basic research and biotechnological purposes. Nevertheless, it is widely known that a prolonged *in vitro* cultivation of plant cells is associated with genetic and epigenetic instabilities, which may limit the usefulness of plant lines. In this study, the age-dependent epigenetic and physiological changes in an asynchronous *Arabidopsis* T87 cell culture were examined. A prolonged cultivation period was found to be correlated with a decrease in the proliferation rate and a simultaneous increase in the expression of senescence-associated genes, indicating that the aging process started at the late growth phase of the culture. In addition, increases in the heterochromatin-specific epigenetic markers, i.e., global DNA methylation, H3K9 dimethylation, and H3K27 trimethylation, were observed, suggesting the onset of chromatin condensation, a hallmark of the early stages of plant senescence. Although the number of live cells decreased with an increase in the age of the culture, the remaining viable cells retained a high potential to efficiently perform photosynthesis and did not exhibit any symptoms of photosystem II damage.

1. Introduction

Plant cell cultures have been demonstrated to be suitable models for studying basic physiological and molecular mechanisms [1,2] as well as plant development, including aging [3]. Additionally, the use of plant cell cultures as tools for producing pharmaceutical proteins, which is considered a 'molecular biofarming' approach, is also being developed [4]. The relatively homogeneous suspension-cultured cells offer certain advantages over whole plants/plant organs, i.e., high reproducibility, fast growth, and simplicity. Although it is widely accepted that plant cell cultures are a less complex model, it is believed that their developmental phases reflect the corresponding phases that occur in a leaf [3].

However, it is also widely known that long-term cell culturing and/or a high number of passages during plant cell culture may result in heritable changes in phenotype resulting from both genetic and epigenetic mechanisms, and these changes are known somaclonal variations [5]. In some cases, these changes are desirable, but some of these changes can also decrease the robustness, reproducibility, growth rate, and/or productivity of the cell lines. Somaclonal variations may thereby impede the usefulness of plant cell lines for biotechnological applications [5].

Although there are many reports concerning the senescence of whole plants/plant organs, only a few studies have focused on age-related changes in plant cell suspension cultures (i.e., [1] and [3]). Consequently, the best known model of plant senescence is the organ-specific degradation of a leaf. This process is characterized by dramatic changes, including a loss of photosynthetic activities as well as changes in the metabolome, transcriptome, and epigenome, which are collectively known as 'leaf senescence syndrome' (reviewed in [6]).

There are some reports focusing on the epigenetic mechanisms involved in leaf senescence (reviewed in [7]); nevertheless, not much is known about the epigenetic age-related changes that occur in suspension-cultured plant cells. A detailed study established that potato cell cultures show a transient decrease in DNA methylation and a simultaneous increase in the acetylation of core

* Corresponding author. Address: Department of Botany, University of Rzeszow, Rejtana 16C, PL 35-959 Rzeszow, Poland.
E-mail address: A.Kwiatkows@gmail.com (A. Kwiatkowska).

http://dx.doi.org/10.1016/j.bbrc.2014.03.141
0006-291X/

histones, and these changes were associated with an increase in transcription rates. All of the observed epigenetic changes appeared, however, after subculture and were reset during the aging of the cell culture [1].

In this study, an asynchronous, photoautotrophic suspension of cultured *Arabidopsis* T87 cells was used as a model for the examination of age-related epigenetic and physiological changes.

2. Materials and methods

All of the diagrams show the means from at least three independent experiments.

2.1. Plant materials

An undifferentiated, photoautotrophic, and asynchronous *Arabidopsis thaliana* T87 cell suspension was cultured as described elsewhere [8] in Gamborg's B5 medium with 15 g/l sucrose and 2.26 μM 2,4-D at 22 °C and 120 rpm on a shaking platform under continuous light conditions. The culture was subcultivated by a tenfold dilution every seven days under standard conditions or maintained for 42 days without subcultivation (long-term cultivation). Cells from the long-term culture were collected on days 7, 14–21, and 42, which correspond to the early, middle, and late phases of cell suspension growth, respectively.

2.2. Protoplast isolation

Because plant suspension-cultured cells tend to form aggregates, the separation of the individual protoplasts is necessary for cell (protoplast) counting and for a better visualization of the silver-stained NORs. Briefly, 10 ml of each culture was centrifuged for 10 min at 300×*g* and 22 °C. The pellet was washed 3 times with mannitol/MES buffer (0.4 M mannitol and 20 mM MES, pH 5.5) and then digested in mannitol/MES buffer supplemented with 1% (w/v) cellulase R10 and 0.1% (w/v) macerozyme R10 (Yakult Pharmaceutical Ind.) for 1–3 h under standard cultivation conditions. The obtained protoplasts were washed 3 times with cold 0.4 M mannitol and resuspended in the same buffer.

2.3. Cell viability

To examine the cell viability, the obtained protoplasts were stained with Trypan blue and counted (TC-10 Automated Cell Counter, Bio-Rad). The cell viability was expressed as a percentage of the viable cells (unstained) in a whole batch.

2.4. Semiquantitative PCR

The total RNA from the cells was isolated as described elsewhere [9], treated with TURBO™ DNAse (Ambion), and reverse transcribed using the iScript™ Select cDNA Synthesis Kit (BIO-RAD) according to the manufacturer's instructions. Semi-quantitative PCR was performed with *Arabidopsis* gene-specific primers: *ATAURORA2*, 5′-TGATTTTGGAGTATGCTGTA-3′ and 5′-ATCTCAGGTGGAAGGTAATC-3′ (the primers are described elsewhere [10]); *EIN2*, 5′-CTTGGCTTCATCGTGCTACA-3′ and 5′-ACCCC AGAAATCCCAAAAAC-3′; *WRKY53*, 5′-AAACTGTTGGGCAACGAAAC-3′ and 5′-AATGGCTGGTTTGACTCTGG-3′; *CAB2*: 5′-GAGGAAGACTG TTGCCAAGC-3′ and 5′-CCCACCTGCTGTGGATAACT-3′; and *actin*, 5′-TCGGTGGTTCCATTCTTGCT-3′ and 5′-GCTTTTTAAGCCTTTGATCT TGAGAG-3′; The amplification was performed under the following conditions, and similar conditions were used for each gene: denaturation at 95 °C for 5 min, 27–30 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s (or 1 min for *AtAurora2*). To ensure that the PCR was linear, the optimal amount of cDNA and the optimal number of cycles were determined for each primer pair. The expression of the investigated genes was normalized to the *actin* transcript levels, and the normalized expression levels are presented.

2.5. Protein isolation and Western blot analysis

The proteins were isolated as described elsewhere [11]. The amount of protein was determined through a densitometric analysis of stained bands resolved by 15% SDS-polyacrylamide gel electrophoresis. The modified isoforms of histone H3 were detected using commercial antibodies from Millipore (anti-phosphoS10-H3, cat. no. 06-570) and Abcam (anti-dimethylK9-H3, cat. no. ab1220; anti-trimethylK27-H3, cat. no. ab6002). The intensities of the Western blot signals were estimated using the ImageJ program and are expressed in arbitrary units per unit amount of protein.

2.6. AgNOR staining and quantification

The protoplasts were fixed in buffer containing 0.4 M mannitol and 20 mM MES (pH 5.5) supplemented with 1% formaldehyde. Twenty microliters of each protoplast sample were placed on microscope slides, air-dried, and permeabilised for 2 min with cold 0.1% sodium citrate supplemented with 0.1% Triton X-100. The transcriptional rDNA activity was assessed as the size of the AgNOR silver deposits, as described elsewhere [12]. The analysis of interphase AgNORs was conducted through the morphometric method using the Olympus CellF software. The interphase NOR activity (area of silver deposits) was expressed as the mean nucleolus-to-nucleus ratio [13,14].

2.7. DNA extraction and methylation quantification

The genomic DNA was extracted as described elsewhere [15] and treated with RNase A. The global DNA methylation was estimated as the 5-methyl-2′-deoxycytidine (5-mdC) level through high-performance liquid chromatography (HPLC) as described elsewhere [16].

2.8. Chlorophyll *a* fluorescence

Ten milliliters of the cell suspension were spread between two layers of highly transparent visible-light polyethylene films and maintained in the dark for at least 30 min. The chlorophyll *a* fluorescence characteristics of the dark-adapted cells were analyzed using a leaf chamber fluorometer (Li-1800-40) fitted with a LI-6400XT portable photosynthesis system (LICOR, US). The minimum fluorescence, F_0, was determined using a modulated pulse of 0.05 μmol photons m^{-2} s^{-1} for 1.8 s. The maximum fluorescence, F_m, was measured after applying a saturating actinic light pulse of 8000 μmol photons m^{-2} s^{-1} for 0.7 s. Based on the above-recorded parameters, both the variable fluorescence, $F_v = (F_m - F_0)$, and the F_v/F_m ratio were calculated. At least five samples from each examined phase of the cell suspension culture were analyzed, and the data were subjected to MANOVA analysis and Duncan's multiple range test ($p < 0.05$) using the Statistica v. 7.1 package.

3. Results and discussion

3.1. Cell viability and proliferation rate

Trypan blue staining showed that the percentage of live cells on day 7 was 95% and then decreased to 73% and 30% on days 14 and 42, respectively (Fig. 1A). Subsequently, the proliferation rate was

determined by an evaluation of the expression of the mitotic kinase *AtAurora2* (Fig. 1B), the mitotically induced and AtAurora-dependent phosphorylation of histone 3 at serine 10 (H3S10ph) (Fig. 1C), and the nucleolus-to-nucleus ratio, which is regarded as a cell-proliferation-activity-related marker. (Fig. 1D). The levels of all of the investigated markers were decreased by approximately 60–70% on day 42 compared with the levels observed on day 7, corresponding to the similar decline in the proliferation rate of the suspension-cultured cells.

Plant Auroras [17,18] are mitotic, cell-cycle-dependent kinases that are maximally expressed at the M phase. *Arabidopsis* Auroras (AtAuroras) are abundant in developing organs, which mainly consist of diving cells, i.e., young roots, flower buds, and flowers [17]. In this study, the *AtAurora2* transcript level was shown to be highest at the early phase of cell suspension growth and then gradually decreased (Fig. 1B). AtAuroras have also been reported to phosphorylate H3S10 *in vitro* [18]. Because Aurora-dependent H3S10 phosphorylation has been detected in plants on a massive scale during active cell division, H3S10ph is believed to be an epigenetic marker of the proliferation rate [19]. Our findings showed a significant decline in H3S10ph with an increase in the age of the *Arabidopsis* T87 suspension (Fig. 1C) and are thus consistent with data obtained by Sokol and colleagues (2007), who observed a similar effect in the same line. According to the authors' explanation, the age-dependent loss of H3S10ph results from the growing number of quiescent and thus non-dividing but still viable cells [2]. Taken together, these data suggest that the number of cells entering mitosis decreases continuously during cell suspension cultivation. However, direct evidence needs to be obtained by, for example, flow cytometry analyses.

Another marker that is believed to be related to cell proliferation activity and to reflect the cellular rDNA transcriptional activity is the size of the interphase nucleolar organiser regions (NORs), which is expressed as nucleolus-to-nucleus ratio [13,14]. NORs are chromosomal segments containing ribosomal genes. Active NORs consist of a set of argyrophilic nucleolar proteins that can

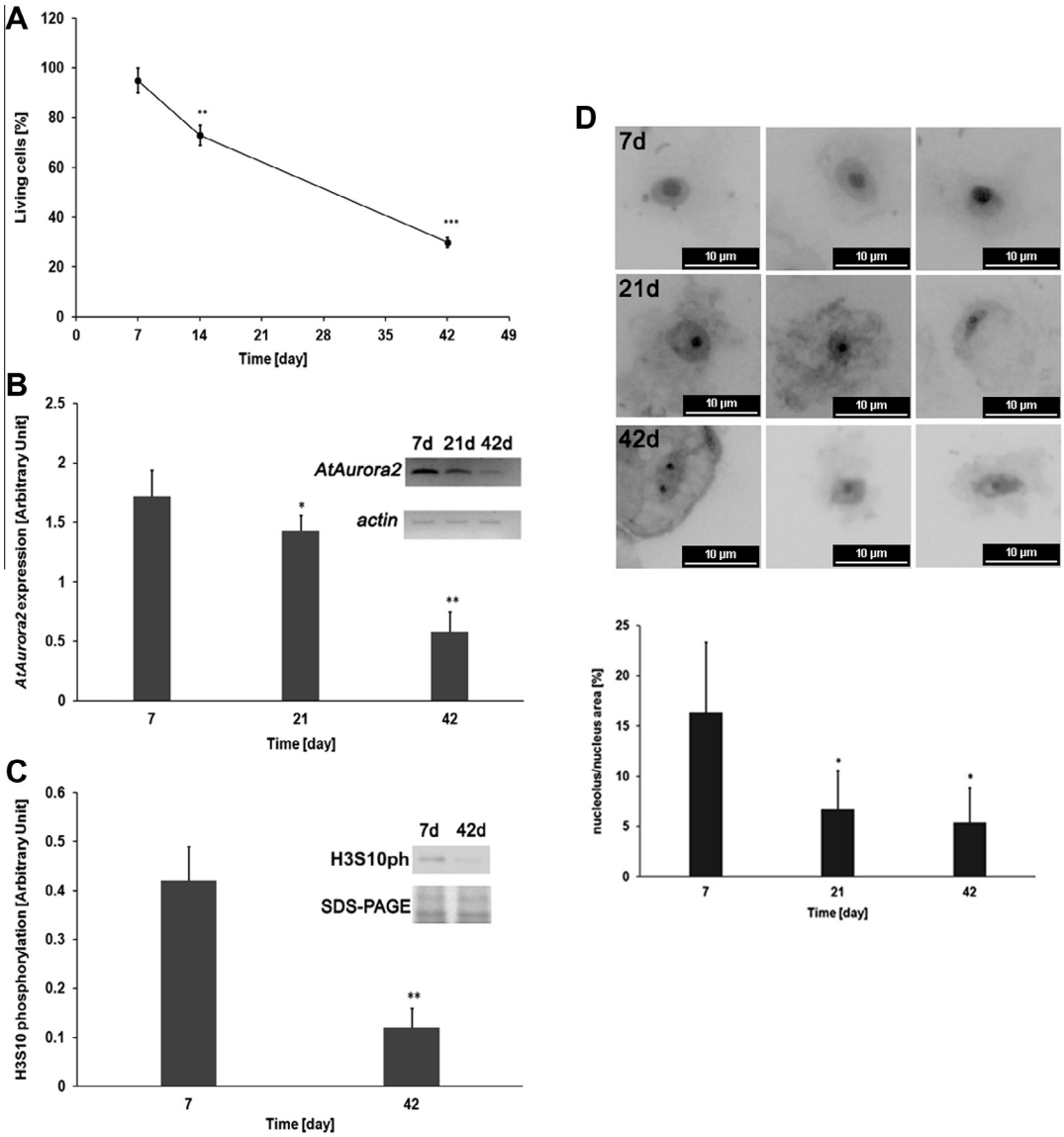

Fig. 1. (A) Cell viability during long-time cultivation of *Arabidopsis* T87 cells (Trypan blue staining). (B–D) The age-dependent changes in the proliferation rate of an *Arabidopsis* cell suspension were evaluated by the *AtAurora2* transcript level (RT-PCR), which was normalized to the *actin* transcript levels (diagram) (B), the H3S10 phosphorylation level (Western blot), which is expressed in arbitrary units per unit amount of protein detected on a Coomassie-stained gel (diagram) (C), and the nucleolus-to-nucleus ratio (after silver nitrate staining), which was calculated using the morphometric method (diagram). The error bars represent the standard deviation of the measurements obtained from at least 40 samples. The images show the typical size of the AgNORs on days 7, 21, and 42 (D).

be easily silver-stained and visualized thereafter as black dots throughout the nucleolar area (designed as AgNORs) [14,20,21]. It is known that a higher abundance of AgNOR proteins is observed in highly proliferating cells compared with non-proliferating cells [20]. Additionally, a successive disappearance of nucleoli during *Arabidopsis* leaf senescence has been reported [22].

In summary, our study shows that both the cell viability and the proliferation rate of long-term cultivated *Arabidopsis* T87 cells decreases significantly with an increase in the age of the culture.

3.2. Expression of senescence-associated genes

To determine the developmental phases of cell suspension, the expression of two well-known positive regulators of plant senescence, namely *Ein2* (ethylene-insensitive 2) and *WRKY53*, was determined. Semiquantitative RT-PCR analysis revealed that the *Ein2* and *WRKY53* transcripts levels are increased approximately 1.7- and 1.3-fold, respectively, from day 7 to day 42 (Fig. 2).

Ein2 is a central component of the ethylene signaling pathway that promotes senescence [23]. Additionally, *Ein2* was recently shown to be involved, along with *miR164*, in a regulatory pathway that upregulates the expression of *ORE1*, another positive regulator of senescence [24]. *WRKY53*, a well-known member of the WRKY transcription factor family, promotes senescence by, for example, regulating the expression of several senescence-associated genes [25].

Taken together, these data indicate that the cell suspension starts aging on 42 day of growth.

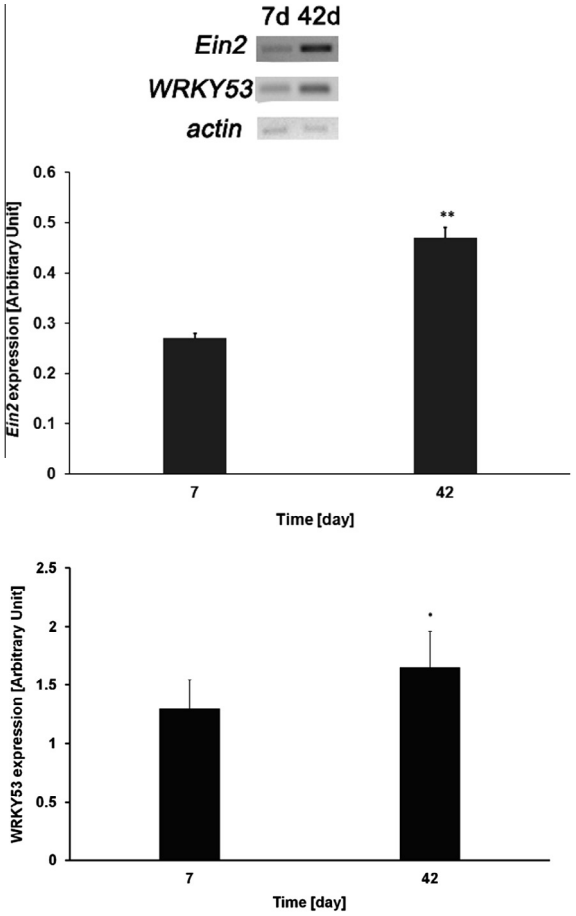

Fig. 2. Age-dependent changes in the expression levels of *Ein2* and *WRKY* (RT-PCR) in *Arabidopsis* T87 cells. The expression levels shown are normalized to the *actin* transcript levels (diagram).

3.3. Heterochromatin-specific epigenetic marks

We then asked whether the epigenetic mechanisms may contribute to the aging process in an *Arabidopsis* suspension culture. Chromatin condensation (heterochromatinization) has been reported as a feature of the early stages of the leaf senescence process [26]. Heterochromatinization is accompanied by an increase in the methylation of DNA and histone 3, particularly H3K9 dimethylation (H3K9me2). Moreover, the level of H3K27 trimethylation

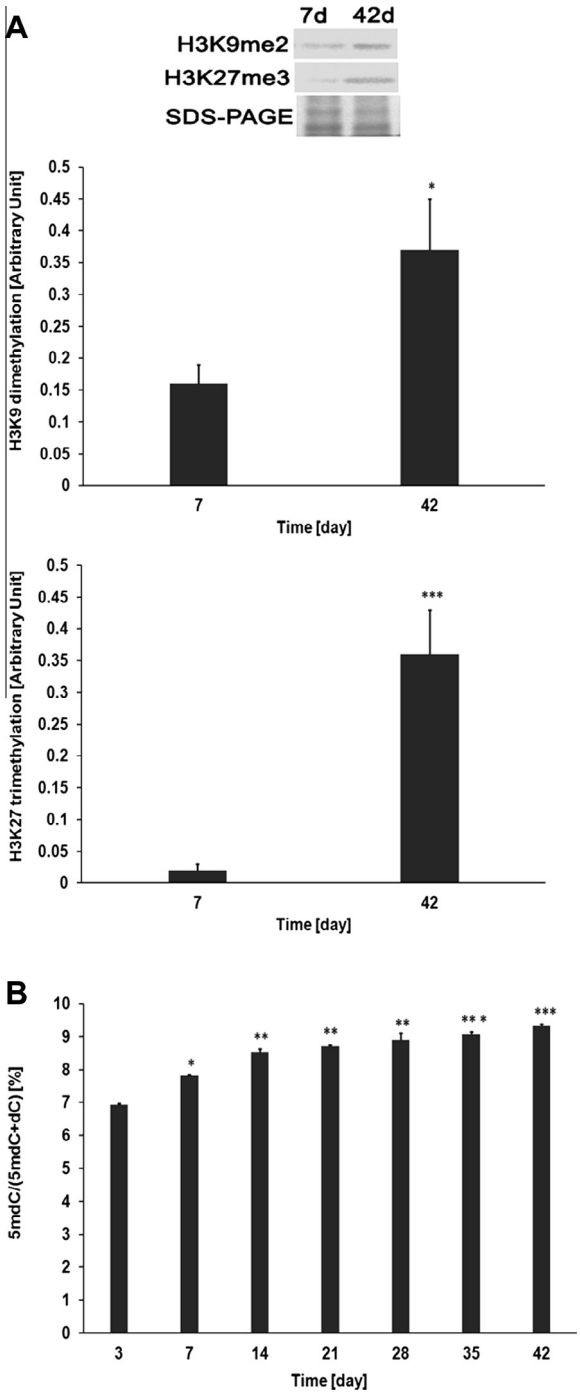

Fig. 3. (A) Age-related changes in H3K27me3 and H3K9me2 (Western blots), which are expressed in arbitrary units per unit amount of protein detected on a Coomassie-stained gel (diagrams). (B) Age-dependent changes in the global DNA methylation level in *Arabidopsis* cells. The global DNA methylation was estimated as the 5-mdC level (HPLC).

(H3K27me3) also plays a role in the repression of genes [27]. Thus, these epigenetic markers were examined in the present study. A Western blot analysis revealed that the levels of global H3K9me2 and H3K27me3 on day 7, were approximately 50% and 95% lower, respectively, than those observed on day 42 (Fig. 3A).

Senescence is accompanied by changes in the pattern of eu- and heterochromatic regions and by the redistribution of heterochromatin-specific markers. For example, at the beginning of senescence, H3K9me2 is detected in larger areas of the nuclei [22]. Another marker that plays a key role in gene repression, mainly by controlling the expression of genes involved in development, is H3K27me3 [27]. A well-known example of this type of epigenetic regulation is the developmental silencing of *Arabidopsis AtTERT*, which encodes a telomerase, associated with an increased loading of H3K27me3 [28]. Our study revealed significant increases in both H3K9me2 and H3K27me3 at the late phase of culture growth. However, further study is needed to determine the gene-specific changes in H3K9me2 and H3K27me3 using ChIP analysis followed by high-throughput methods (i.e., sequencing or microarrays technologies).

Heterochromatinization is also associated with DNA methylation. Our study revealed that the global DNA methylation, which was evaluated by the 5-mdC content, was approximately 26% lower on day 7 compared with day 42 (Fig. 3B). It is known that plant nuclear DNA, in contrast to animal nuclear DNA, is highly methylated and that this modification is species-, tissue-, organelle-, and age-specific. DNA in plants can be methylated at CG as well as non-CG sequences, i.e., CHG and CHH (where H is A, T, or C) [29]. Nevertheless, very little is known about the epigenetic changes that accompany aging in plant suspension-cultured cells.

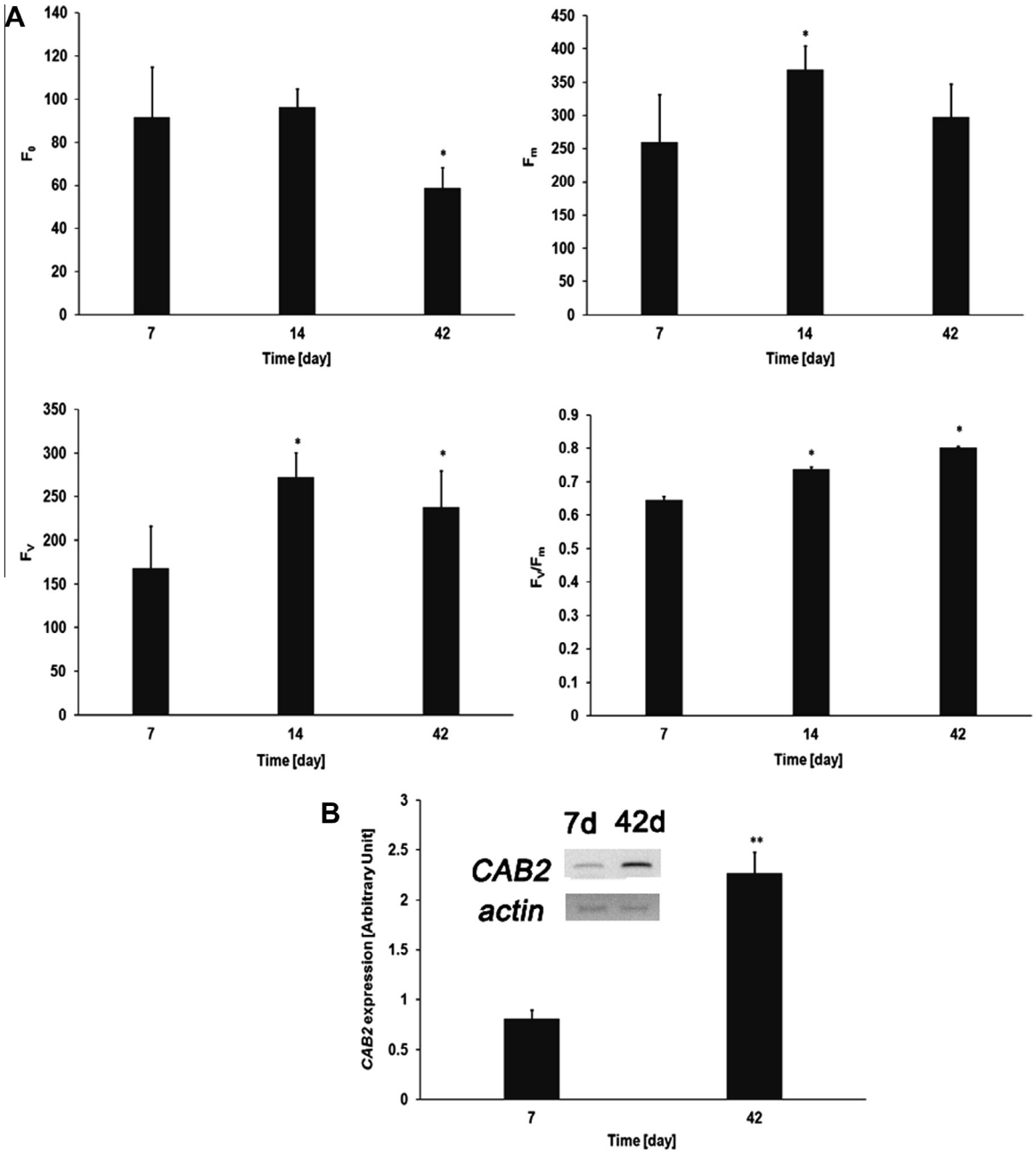

Fig. 4. Changes in the photosynthesis-related markers with respect to the age of an *Arabidopsis* cell suspension culture. (A) The following chlorophyll *a* fluorescence parameters were determined for dark-adapted cells: F_0, F_m, F_v, and F_v/F_m ratio. (B) Age-dependent changes in the expression level of *CAB2* (RT-PCR). The expression of *CAB2* was normalized to the *actin* transcript levels (diagram).

To the best of our knowledge, only one study has determined that the dynamic control of the 5mC level in non-CG sequences plays a role in the regulation of the growth of an asynchronous potato suspension culture [1]. The authors revealed that the DNA methylation level decreases by one-third due to subculture and then gradually returns to its initial value [1]. The results obtained by both Law and Shuttle [1] and our group indicate the role of DNA methylation in the growth and aging of plant cells. Nevertheless, further investigations are necessary to determine both the gene- and DNA sequence-specific (CG or non-CG) changes in methylation during the long-term cultivation of *Arabidopsis* cultures.

In summary, the increase in heterochromatin-specific markers noted in this study may be associated with age-related chromatin condensation.

3.4. Chlorophyll a fluorescence

To identify the physiological markers of cells in a suspension culture in the context of senescence, we examined four chlorophyll fluorescence characteristics of the dark-adapted cells (Fig. 4A). The minimum fluorescence, F_0, was found to only slightly increase from a value of 91 to a value of 96 from day 8 to day 15 but decreased considerably to a value of 58 on day 42. The F_0 characterizes the maximal photochemistry of photosystem II (PSII), which corresponds to the primary quinone electron acceptor Q_A being in the fully oxidized state [30]. The maximum fluorescence, F_m, increased from 260 to 368 between the day 8 and the day 15, then declined to 297 on the day 42. This parameter corresponds, in turn, to the light saturating stage of PSII, when the first stable electron acceptor Q_A is fully reduced. The variable fluorescence, F_v, which is defined as the difference between F_m and F_0, increased significantly from day 8 to day 15 and then remained constant. Additionally, the F_v/F_m ratio, which is widely accepted as an estimate of the maximum quantum efficiency of PSII photochemistry, substantially increased from 0.645 to 0.738 during the period from day 8 to day 15 and then further increased to 0.802 on day 42.

The four chlorophyll fluorescence parameters examined in the present study, namely, F_0, F_m, F_v, and F_v/F_m ratio, have been widely used as indicators for the early and non-destructive detection of biotic and abiotic stresses [31–33] and to some extent have been employed in the assessment of senescence processes in the leaves of various species [34,35]. Our study shows that all of the parameters are sensitive to the developmental changes of the cells in the suspension culture; however, their alteration in subsequent culture stages depends on the parameter. The F_0 exhibited the best fit with the changes in the genetic markers, namely the expression of *Ein2 and WRKY53* (Fig. 2) and satisfactorily reflected the development of cell senescence. In green leaves, this parameter usually increases in response to environmental stresses, contributing to a reduction of the F_v/F_m ratio [30]. However, F_0 may also depend on the chlorophyll *a* content in a sample, as has been experimentally proven for single-cell plankton organisms using various light wavelength excitations [36]. The cell suspension examined in our study somewhat resembled a pool of chlorophyll in a plankton sample, and the decrease in the F_0 may thus be reasonably considered an index of the declined amount of chlorophyll. Therefore, the decrease in F_0 may correspond to the increase in the dead cell fraction in the cell suspension between day 15 and day 42. The pattern of F_0 alteration obtained in our experiments is consistent with that observed during potato plant aging [37]. The F_v/F_m ratio did not decrease during the tested period, suggesting that the remaining live cells exhibited high potential to efficiently perform photosynthesis and did not exhibit any symptoms of PSII damage on the 42nd day of culture. Similar observations have been reported from the study of the autumnal senescence of leaves from the deciduous tree *Platanus occidentalis* L. [38].

In accordance with these results, our data show a threefold increase in the expression of *CAB2* (chlorophyll a/b-binding protein 2), a PSII-associated component of the light harvesting complex, on day 42 compared with that observed on day 7 (Fig. 4B). Although *CAB2* expression has been found to decrease during senescence [39], an increase in its expression, as was observed in this study, may confirm the high photosynthetic efficiency of the viable cells.

To the best of our knowledge, this study provides the first demonstration of several age-related characteristics of *Arabidopsis* T87 cell culture. Plant cell suspensions can be considered whole multicellular organisms that pass through subsequent phases of development, including aging [3]. Nevertheless, plant suspension cultures enable researchers to bypass the complexity characterizing whole plants and obtain data that are easier to interpret but that still correspond to the data that would be obtained from whole plants.

Acknowledgments

The authors would like to thank Andrzej Jerzmanowski, Krzysztof Kokoszka, Maciej Kotlinski, and Antoni Palusinski (Laboratory of Plant Molecular Biology, University of Warsaw, Poland and Institute of Biochemistry and Biophysics. PAS, Poland) for providing the *Arabidopsis* T87 cell line.

References

[1] R.D. Law, J.C. Suttle, Chromatin remodeling in plant cell culture: patterns of DNA methylation and histone H3 and H4 acetylation vary during growth of asynchronous potato cell suspensions, Plant Physiol. Biochem. 43 (2005) 527–534.
[2] A. Sokol, A. Kwiatkowska, A. Jerzmanowski, M. Prymakowska-Bosak, Up-regulation of stress-inducible genes in tobacco and Arabidopsis cells in response to abiotic stresses and ABA treatment correlates with dynamic changes in histone H3 and H4 modifications, Planta 227 (2007) 245–254.
[3] W. Peters, J. Ritter, H. Tiller, O. Valdes, U. Renner, M. Fountain, E. Beck, Growth, ageing and death of a photoautotrophic plant cell culture, Planta 210 (2000) 478–487.
[4] F.S. Shadwick, P.M. Doran, Foreign protein expression using plant cell suspension and hairy root cultures, in: Molecular Farming, Wiley-VCH Verlag GmbH & Co. KGaA, 2005, pp. 13–36.
[5] S. Mohan Jain, Tissue culture-derived variation in crop improvement, Euphytica 118 (2001) 153–166.
[6] V. Buchanan-Wollaston, Senescence processes in plants. Annual plant reviews, volume 26, Ann. Bot. 101 (2008) 197.
[7] K. Humbeck, Epigenetic and small RNA regulation of senescence, Plant Mol. Biol. 82 (2013) 529–537.
[8] M. Axelos, C. Curie, C. Bardet, B. Lescure, A protocol for transient expression in *Arabidopsis thaliana* protoplasts isolated from cell suspension cultures, Plant Physiol. Biochem. 30 (1992) 123–128.
[9] N. Etheridge, Y. Trusov, J. Verbelen, J. Botella, Characterization of ATDRG1, a member of a new class of GTP-binding proteins in plants, Plant Mol. Biol. 39 (1999) 1113–1126.
[10] D. Kurihara, A. Kawabe, S. Matsunaga, K. Nakagawa, S. Fujimoto, S. Uchiyama, K. Fukui, Characterization of a splicing variant of plant aurora kinase, Plant Cell Physiol. 48 (2007) 369–374.
[11] M. Tariq, H. Saze, A.V. Probst, J. Lichota, Y. Habu, J. Paszkowski, Erasure of CpG methylation in Arabidopsis alters patterns of histone H3 methylation in heterochromatin, Proc. Natl. Acad. Sci. 100 (2003) 8823–8827.
[12] W.M. Howell, D.A. Black, Controlled silver-staining of nucleolus organizer regions with a protective colloidal developer: a 1-step method, Experientia 36 (1980) 1014–1015.
[13] M. Munke, H. Schmiady, K. Sperling, Ag-staining of nucleolus organizer regions on human prematurely condensed chromosomes from cells with different ribosomal RNA gene activity, Exp. Cell Res. 121 (1979) 425–428.
[14] M. Derenzini, The AgNORs, Micron 31 (2000) 117–120.
[15] J.J. Doyle, J.L. Doyle, A rapid total DNA preparation procedure for fresh plant tissue, Focus 12 (1990) 13–15.
[16] L. Potocki, A. Lewinska, J. Klukowska-Rötzler, A. Bielak-Zmijewska, W. Grabowska, I. Rzeszutek, P. Kaminska, E. Roga, M. Bugno-Poniewierska, E. Slota, K. Mählmann, C. Koch, M. Wnuk, Sarcoid-derived fibroblasts: links between genomic instability, energy metabolism and senescence, Biochimie 97 (2014) 163–172.
[17] D. Demidov, D. Van Damme, D. Geelen, F.R. Blattner, A. Houben, Identification and dynamics of two classes of Aurora-like kinases in arabidopsis and other plants, Plant Cell 17 (2005) 836–848.

[18] A. Kawabe, S. Matsunaga, K. Nakagawa, D. Kurihara, A. Yoneda, S. Hasezawa, S. Uchiyama, K. Fukui, Characterization of plant Aurora kinases during mitosis, Plant Mol. Biol. 58 (2005) 1–13.
[19] A. Houben, T. Wako, R. Furushima-Shimogawara, G. Presting, G. Künzel, I. Schubert, K. Fukui, The cell cycle dependent phosphorylation of histone H3 is correlated with the condensation of plant mitotic chromosomes, Plant J. 18 (1999) 675–679.
[20] V. Sirri, P. Roussel, D. Hernandez-Verdun, The AgNOR proteins: qualitative and quantitative changes during the cell cycle, Micron 31 (2000) 121–126.
[21] D. Trerè, AgNOR staining and quantification, Micron 31 (2000) 127–131.
[22] N. Ay, K. Irmler, A. Fischer, R. Uhlemann, G. Reuter, K. Humbeck, Epigenetic programming via histone methylation at WRKY53 controls leaf senescence in *Arabidopsis thaliana*, Plant J. 58 (2009) 333–346.
[23] J.M. Alonso, T. Hirayama, G. Roman, S. Nourizadeh, J.R. Ecker, EIN2, a bifunctional transducer of ethylene and stress responses in Arabidopsis, Science 284 (1999) 2148–2152.
[24] J.H. Kim, H.R. Woo, J. Kim, P.O. Lim, I.C. Lee, S.H. Choi, D. Hwang, H.G. Nam, Trifurcate feed-forward regulation of age-dependent cell death involving miR164 in Arabidopsis, Science 323 (2009) 1053–1057.
[25] Y. Miao, T. Laun, P. Zimmermann, U. Zentgraf, Targets of the WRKY53 transcription factor and its role during leaf senescence in Arabidopsis, Plant Mol. Biol. 55 (2004) 853–867.
[26] I. Kolodziejek, J. Koziol-Lipinska, M. Waleza, J. Korczynski, A. Mostowska, Aspects of programmed cell death during early senescence of barley leaves: possible role of nitric oxide, Protoplasma 232 (2007) 97–108.
[27] P. Fransz, H. de Jong, From nucleosome to chromosome: a dynamic organization of genetic information, Plant J. 66 (2011) 4–17.
[28] A. Ogrocká, E. Sýkorová, J. Fajkus, M. Fojtová, Developmental silencing of the AtTERT gene is associated with increased H3K27me3 loading and maintenance of its euchromatic environment, J. Exp. Bot. 63 (2012) 4233–4241.
[29] B.F. Vanyushin, V.V. Ashapkin, DNA methylation in higher plants: past, present and future, Biochim. Biophys. Acta 2011 (1809) 360–368.
[30] K. Maxwell, G.N. Johnson, Chlorophyll fluorescence – a practical guide, J. Exp. Bot. 51 (2000) 659–668.
[31] N.R. Baker, E. Rosenqvist, Applications of chlorophyll fluorescence can improve crop production strategies: an examination of future possibilities, J. Exp. Bot. 55 (2004) 1607–1621.
[32] C. Rousseau, E. Belin, E. Bove, D. Rousseau, F. Fabre, R. Berruyer, J. Guillaumès, C. Manceau, M.-A. Jacques, T. Boureau, High throughput quantitative phenotyping of plant resistance using chlorophyll fluorescence image analysis, Plant Methods 9 (2013) 1–13.
[33] H. Kalaji, V. Goltsev, K. Bosa, S. Allakhverdiev, R. Strasser, Govindjee, Experimental in vivo measurements of light emission in plants: a perspective dedicated to David Walker, Photosynth. Res. 114 (2012) 69–96.
[34] D. Panda, R.K. Sarkar, Natural leaf senescence: probed by chlorophyll fluorescence, CO2 photosynthetic rate and antioxidant enzyme activities during grain filling in different rice cultivars, Physiol. Mol. Biol. Plants 19 (2013) 43–51.
[35] A. Wingler, M. Marès, N. Pourtau, Spatial patterns and metabolic regulation of photosynthetic parameters during leaf senescence, New Phytol. 161 (2004) 781–789.
[36] G. Parésys, C. Rigart, B. Rousseau, A.W.M. Wong, F. Fan, J.P. Barbier, J. Lavaud, Quantitative and qualitative evaluation of phytoplankton communities by trichromatic chlorophyll fluorescence excitation with special focus on cyanobacteria, Water Res. 39 (2005) 911–921.
[37] G. Mauromicale, A. Ierna, M. Marchese, Chlorophyll fluorescence and chlorophyll content in field-grown potato as affected by nitrogen supply, genotype, and plant age, Photosynthetica 44 (2006) 76–82.
[38] W.W. Adams, K. Winter, U. Schreiber, P. Schramel, Photosynthesis and chlorophyll fluorescence characteristics in relationship to changes in pigment and element composition of leaves of *Platanus occidentalis* L. during autumnal leaf senescence, Plant Physiol. 92 (1990) 1184–1190.
[39] V. Buchanan-Wollaston, Isolation of cDNA clones for genes that are expressed during leaf senescence in *Brassica napus* (Identification of a gene encoding a senescence-specific metallothionein-like protein), Plant Physiol. 105 (1994) 839–846.