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RESEARCH PAPERS

Cytophysiological Characteristics of *Arabidopsis thaliana* Cultivated Cells with Disable Perception of Ethylene Signal by the ETR1 Receptor

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Abstract—Contradictory data about ethylene influence on cell growth and division prompted us to investigate cytophysiological characteristics of suspension cultures of *Arabidopsis thaliana* of wild type Col-0 and *ert1-1* mutant carrying a point mutation in the site of ethylene binding by the ETR1 receptor. Some cytophysiological characteristics of the *etr1-1* cultivated cells differed from those of Col-0: the growth rate of mutant cells was less and cell sizes were smaller, the culture was committed to the formation of tracheary elements (TE), had a pronounced modal class of nuclei (54%) with the amount of DNA 8C and a tendency to expand the ploidy toward 32C. Despite the absence of ethylene perception by the ETR1 receptor, the cell culture of mutant responded to treatment with ethylene by growth acceleration, an increase in cell viability and in the number of cells in the S-phase of the cell cycle. The inhibitor of ethylene binding to receptors, 1-methylcyclopropene, suppressed growth and viability of the cells of both genotypes. In the *etr1-1* cell culture, the inhibitor reduced the number of S-phase nuclei and activated TE formation. All data obtained indicate that ethylene perception and transduction of ethylene signal are required for the maintenance of cell viability and active in vitro growth. It is supposed that the functional activity of the ETR1 receptor is necessary for optimal cell expansion, whereas other receptors are responsible for cell proliferation.

Keywords: Arabidopsis thaliana, etr1-1, ethylene, 1-methylcyclopropene, cell culture, growth, S-phase, tracheary elements, DNA cytophotometry

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INTRODUCTION

Gaseous phytohormone ethylene regulates seed germination, climacteric fruit ripening, cell expansion, root hair formation, leaf and flower senescence and abscission, and the responses to biotic and abiotic stresses [1]. As distinct from other classic phytohormones, ethylene and the inhibitors of its action are widely applied in practice. The study on molecular mechanisms of ethylene action became possible due to the identification in *Arabidopsis thaliana* plants the components of the signaling pathway from ethylene perception by its receptors to the ethylene-dependent gene expression [2, 3].

Ethylene receptors were identified by the molecular-genetic analysis of mutations leading to the appearance of ethylene-insensitive phenotypes [4–8]. All these mutations are dominant and are provoked by the point replacements of nucleotide bases. Thus, in the *etr1-1* mutant (from ethylene resistant), Cys65 is replaced by Tyr in the second transmembrane domain of the ethylene-binding site of the ETR1 receptor [5]. Since Cu^{2+} cations are involved in the functioning of high-affinity ethylene binding site upon interaction with conserved amino acid residues Cys65 and His69, the *etr1-1* mutant lost a capability of ethylene binding, being incapable to coordinate Cu^{2+} cations. In addition to the *etr1-1*, there are three allele dominant point mutations in *ETR1* affecting ethylene binding by the receptor. Thus, in yeast-expressed *etr1-2* with a mutation in the third transmembrane domain, ethylene binding was by 50% higher than in yeast expressing wild-type *ETR1* gene [9]. Hence, the insensitivity to ethylene of some *Arabidopsis* "receptor mutants," but not *etr1-1*, is related not to the loss of the receptor capability to bind ethylene but rather to the loss of the capability of signal transduction.

Further studies showed that in *Arabidopsis* there is a small family of proteins localized in the endoplasmic reticulum, which structure is close to that of ETR1 [10]. The highest degree of homology is characteristic of the molecule regions responsible for ethylene binding. In addition to ETR1 this family comprised ERS1, ETR2, EIN4, and ERS2 proteins, which are identical to ETR1 by 60–65% [6–8]. The family of ethylene

Abbreviations: 2C—DNA amount in the diploid chromosome set; EdU—5-ethynyl-2'-deoxiuridine; 1-MCP—1-methylcyclopropene; PBS—phosphate buffered saline; TE—tracheary elements.

receptors is subdivided into the two subfamilies. The subfamily I includes ETR1 and ERS1 proteins with three transmembrane domains in the N-terminal part of the macromolecule and a histidine kinase domain in the C-terminal region. Proteins of the subfamily II, ETR2, ERS2, and EIN4, comprise four transmembrane domains and Ser/Thr protein kinase domain in the C-terminal region [10].

Since in etiolated seedlings of dominant receptor mutants *etr1, ers1, etr2, ers2*, and *ein4*, "triple response" to ethylene was absent, the idea arose that functions of ethylene receptors are overlapped [4, 6–8]. This view remained firm until obtaining and detail studying the plants with a recessive mutation in the *ERS1* gene [11, 12]. Recent studies confirmed a possibility that ethylene receptors can not only replace each other as negative regulators of triple response to ethylene but also manifest individual properties required for the specific responses to ethylene. At the same time, all available data indicate that *Arabidopsis* receptors of the subfamily I (ETR1 and ERS1) play the more important role in triggering ethylene signal transduction [10].

It has been shown long ago that, at the work with isolated cultures of cells and tissues of various plant species and under various growing conditions, the concentration of ethylene in the culturing flasks increased up to several tens of ppm (μL/L) [13]. At the same time, it is well known that physiological effects of ethylene are manifested at lower by two–three orders concentrations [1]. Does excess of ethylene affect cultured cells and how? This question was raised many times, and the answers were often contradictory: from stimulation of callus growth to inhibition of cell suspension culture growth [13].

In this connection, the objective of this work was to study cytophysiological characteristics of cultured *Arabidopsis thaliana* wild-type cells and those of *etr1-1* mutant with a mutated ethylene receptor *ETR1* gene.

MATERIALS AND METHODS

Plant material. Experiments were performed with suspension cultures of *Arabidopsis thaliana* (L.) Heynh. cells of two genotypes: wild type (ecotype Columbia, Col-0) and *etr1-1* mutant. Seeds of Col-0 plants [N1092] and *etr1-1* mutant [N237] were purchased from the Nottingham *Arabidopsis* Stock Centre (NASC, Great Britain). For callus formation, completely developed leaves of sterile four-week-old plants were used. Primary callus was grown on agar-solidified MS medium; the most friable callus parts were taken and transferred to the liquid Schenk and Hildebrandt nutrient medium (SH medium) [14].

Suspension cell cultures were grown in 50 mL of SH medium supplemented with 3% sucrose, 1 mg/L 2,4-D, and 0.1 mg/L kinetin in glass 250-mL flasks covered with aluminum foil and Craft paper in darkness at 26°C and continuous stirring (120 rpm). The period of subculturing was 10 days, the inoculate volume was 3 mL for Col-0 and 5 mL for *etr1-1*.

Growth characteristics. Cell suspensions were grown in the series of flasks. During culturing, three flasks were periodically taken and cell fresh weight, their number and sizes were determined. The number of cells was estimated by the number of protoplasts, which were obtained by mixing the equal volumes of the cell suspension and solution containing SH medium macronutrients, 0.8 M sorbitol, 8 mM $CaCl_2$, 25 mM Mes–KOH (pH 5.7), 2% cellulase Onozuka R10 (Kinki Yakult, Japan), 0.3% pectinase Macerozyme R10 (Kinki Yakult), and 0.8% hemicellulase Driselase (Fluka, United States). Protoplasts were isolated at 26°C on the shaker (120 rpm) during 1.0–1.5 h. The number of protoplasts was counted in the Fuchs–Rosental chamber. Protoplasts were photographed, and their diameters were measured.

Counting the number of tracheary elements (TE) in the samples of the *etr1-1* cell suspension was performed after protoplast isolation. An aliquot of protoplast suspension was washed twice with 0.5 M sorbitol with centrifugation at 150 *g* for 10 min. Protoplasts were lysed in 1% Triton X-100 at 37°C, and the number of TE was counted under light microscope.

Cell viability was evaluated by the number of cells unstained with aqueous 0.02% Erythrosin B (Sigma-Aldrich, United States).

Cytophotometric determination of the nuclear DNA content. The cells were fixed with the mixture of glacial acetic acid and 96% ethanol (1 : 3) and washed three times with 70% ethanol for 5 min each. The material was stained according to the standard protocol suggested by Greilhuber [15]. Then squashed preparations were made, and after dehydration in the series of alcohols and xylene they were embedded into DePeX (Serva, Germany). Cytophotometric analysis was performed as has been described earlier [16] by the two-wave method at $\lambda_1 = 502$ nm and $\lambda_2 = 565$ nm. To calculate the ploidy of cell nuclei in suspension cultures on the basis of the DNA content, a standard was used: prophasic (4C) nuclei of the *Vigna radiata* cv. Berken cells of the root apical meristem with known DNA content (4C = 2.1 pg) and also data about DNA content in diploid nuclei of *A. thaliana* cells (2C = 0.32–0.40 pg) presented in the database of Kew Botanical garden (http://data.kew.org/cvalues).

Revealing the cells in the S-phase of the mitotic cycle was performed by the modified method of Kotogány et al. [17]. Samples of cultivated cells (5–6 mL) were taken, the analog of thymidine 5-ethynyl-2'-deoxyuridine (EdU) at the concentration of 20 μM was added, and samples were incubated in darkness at 26°C on the shaker. In 30 min, 200 μM thymidine was added to stop EdU incorporation into DNA. In 10 min, the samples were centrifuged at 500 *g* for 5 min at 4°C, the supernatant was discarded, and the cells were fixed in 4% formalin in phosphate buffered saline (PBS) with 0.1% Triton X-100 for 15 min. Thereafter,

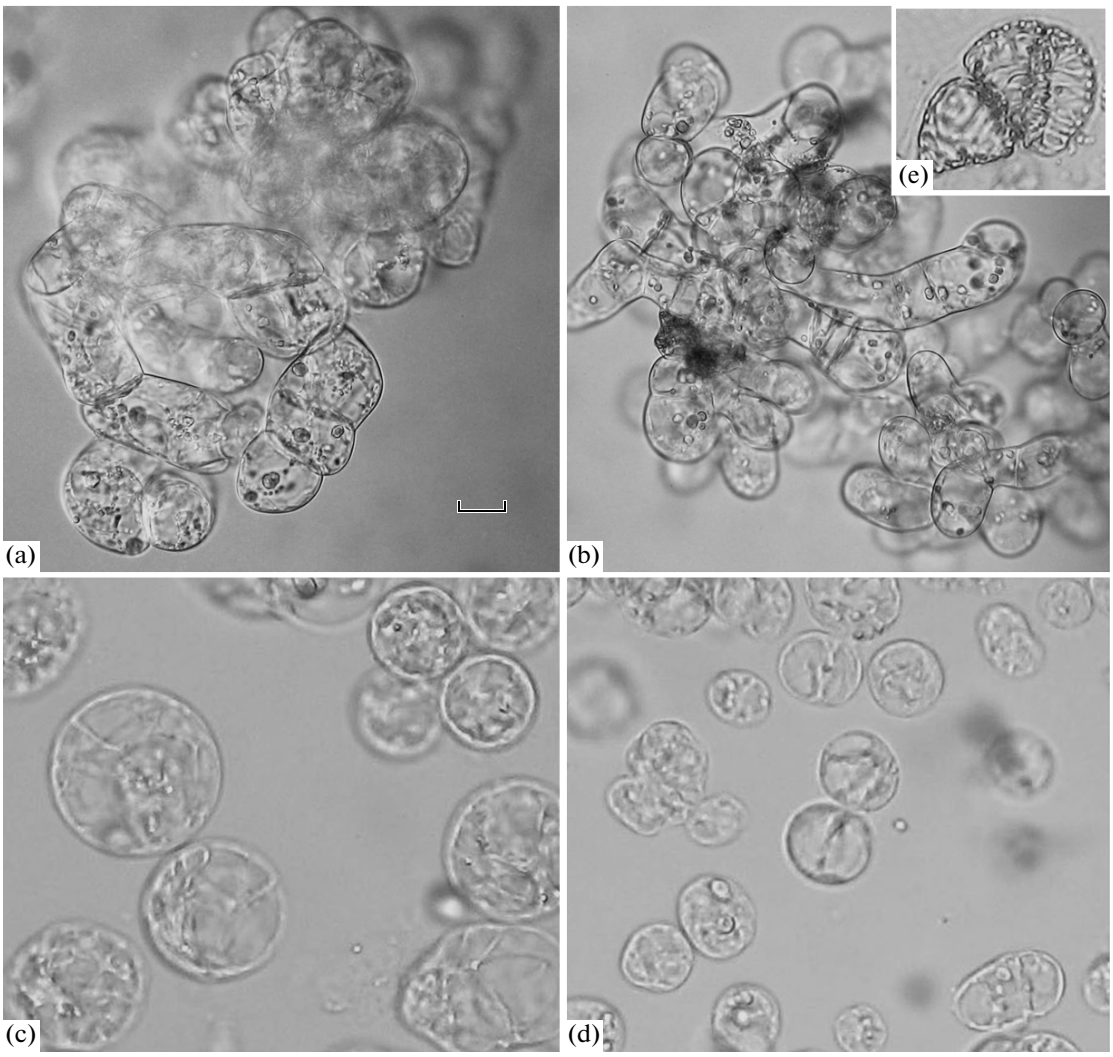

Fig. 1. Microphotographs of cell aggregates of suspension cultures of *A. thaliana* Col-0 (a) and *etr1-1* (b) and protoplasts isolated from cells (c) and (d), respectively.

Tracheary elements developed in the *etr1-1* cell culture are shown in the insert (e). Bar scales are 20 μm on all photos.

the cells were washed three times in PBS, and aliquots (20–30 μL) of cell packed volume were retained in tubes. EdU incorporation was determined in the reaction with Alexa Fluor 488 azide, using Click-iT® EdU Imaging Kit (Invitrogen, Life Technologies, United States). The reaction mixture prepared in accordance with the manufacturer's recommendations (150 μL) was added into each tube. After staining for 30 min (at room temperature in darkness), the cells were washed with PBS supplemented with 2 mM sodium azide, then with PBS and PBS with 100 ng/mL DAPI (Sigma-Aldrich) to stain nuclei. Squashed preparations were prepared and analyzed under fluorescent microscope at 420 nm for DAPI and 510 nm for Alexa Fluor 488.

Ethylene production and determination of the contents of ethylene, carbon dioxide, and oxygen were performed by the methods described earlier [18]. Ethylene was produced at 2-chloroethylphosphonic acid decomposition under alkaline conditions. Ethylene quantification was performed using a Tsvet 106 gas chromatograph (Russia) with a flame ionization detector. Carbohydrates were concentrated in the Porapak N column (80–100 mesh, 70 × 4 mm, Supelko, United States) at –30°C. After desorption at 50°C, ethylene was determined in the Porapak N column (80–100 mesh, 3 m × 2 mm). The contents of carbon dioxide and oxygen were controlled by gas-absorption chromatography.

To measure the rate of ethylene production, aliquots (2.5 mL) were taken from the flasks for cell cultivation and transferred in the glass vials of known volumes. These vials with cells were sealed with self-sealing rubber stoppers (Suba-Seal septa red rubber, Sigma-Aldrich) and incubated for 30 min at 26°C in darkness on the shaker. The vials with medium but without cells

Table 1. Distribution of protoplasts (%) on sizes in the middle of the exponential growth phase of Col-0 and *etr1-1* cell suspension cultures

Protoplast diameter, μm	Col-0 cell culture	*etr1-1* cell culture
8–11	0	1.3 ± 0
11–14	9.0 ± 1.6	26.2 ± 14.6
14–17	11.1 ± 0.8	29.4 ± 2.6
17–20	33.1 ± 6.8	32.2 ± 6.4
20–23	16.8 ± 3.7	4.9 ± 1.0
23–26	12.1 ± 2.8	3.2 ± 2.7
26–29	12.6 ± 2.1	2.8 ± 2.1

Mean values and their standard deviations are presented

were used as control (the ethylene content in air). The gas phase (1–2 mL) was taken from these vials with a syringe for analyses. The gas samples were also taken directly from the flasks and gas bells at different times of subculturing.

1-Methylcyclopropene (1-MCP) was produced by the method of Sisler and Serek [19]. 1-MCP concentration was determined in the gas chromatograph with flame ionization detector by comparing the area of 1-MCP peak and that of known amount of butane. The chromatographic analysis was performed in the Porapak N column (80–100 mesh, 3 m × 2 mm) at 120°C, and the rate of the carrier gas helium was of 60 mL/min.

The effects of ethylene and 1-MCP on cytophysiological characteristics of suspension cultures were observed in cultures grown in 100-mL Erlenmeyer flasks. The suspensions of Col-0 (1.5 mL) and *etr1-1* mutant (2.5 mL) were inoculated into 25 mL of SH medium. Flasks were covered with a single layer of dense chromatographic paper, placed on the table from organic glass under the 20-L gas bell, and sealed. Through tubing attached to the fitting coming out of the table, the following gases were introduced into the bell: 1-MCP (100 nL/L), ethylene (20 μL/L), and the mixture of 1-MCP (100 nL/L) and ethylene (20 μL/L). Control bell contained air. The whole construction was placed on the shaker (26°C, darkness). Airing and gas (1-MCP and ethylene) replacement were performed every four days of cultivation.

To verify the mutation preservation in the genome of cultured *etr1-1* cells, allele-specific PCR was carried out periodically, as described previously [20].

Figures present mean values from three independent replications and their standard deviations.

RESULTS

The stabilization of growth parameters of suspension cultures of Col-0 and *etr1-1* cells occurred during first 1.5 years of cultivation: the size of cell aggregates decreased and the growth rate increased. During the last five years these cultures had stable growth characteristics and cytomorphology. It is important that the genome of the *etr1-1* mutant preserved a point mutation, which was repeatedly demonstrated by the method of allele PCR (see [20]) (data not shown).

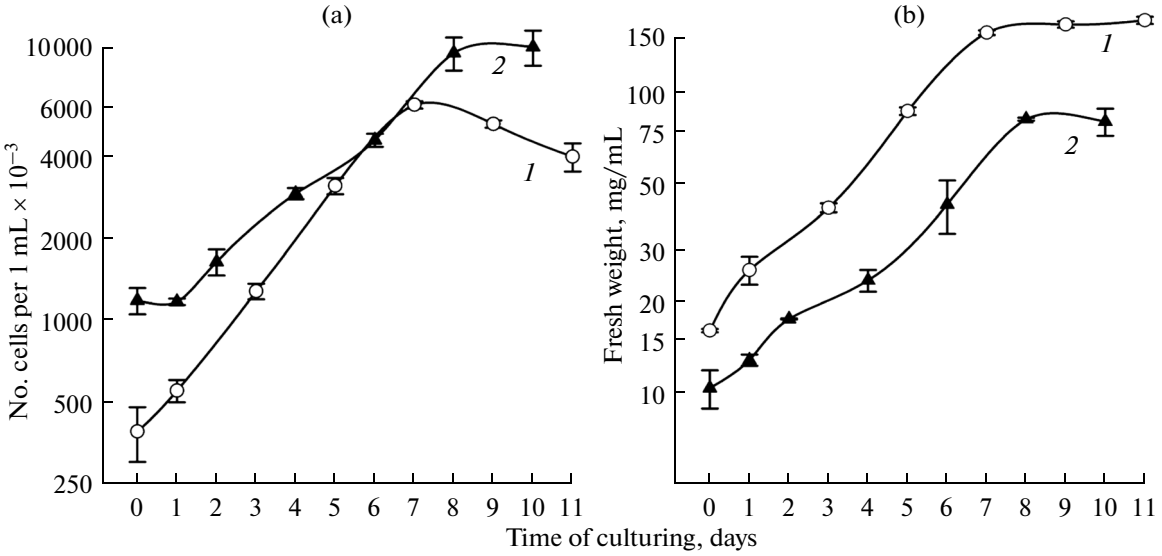

Fig. 2. The time-course of the increase in the number of cells (a) and fresh weight (b) of suspension cultures of *A. thaliana* Col-0 (*1*) and *etr1-1* (*2*) during subculturing.

Ordinate axes are represented in a logarithmic scale.

Table 2. The number of Col-0 and *etr1-1* cells in suspension cultures per 1 mg of fresh weight during subculturing

Time of culturing, days	Col-0 cell culture	*etr1-1* cell culture
0	24846 ± 1038	103222 ± 21368
1	–*	90440 ± 5764
3	33528 ± 3956	–*
4	–*	122234 ± 6779
5	37133 ± 2114	–*
6	–*	110020 ± 28958
7	40585 ± 2716	–*
8	–*	116343 ± 18258
9	25225 ± 8027	–*

Mean values and their standard deviations are presented.
* Index was not determined.

Suspensions formed small cell aggregates, as a rule from 10–15 cells of round or elongated shape; cell files were frequently met (Fig. 1). An important property of the *etr1-1* culture is a capability of tracheary elements (TE) formation, which persisted throughout the all years of in vitro cultivation (Fig. 1e). For the evaluation of culture growth after the number of cells, we used protoplasts (Figs. 1c, 1d); their sizes were measured in parallel. The cells of the *etr1-1* mutant were much smaller than the cells of Col-0. More than 40% of protoplasts isolated from Col-0 cells had a diameter > 20 μm, whereas in the *etr1-1* culture – only about 10% (Table 1, Figs. 1c, 1d).

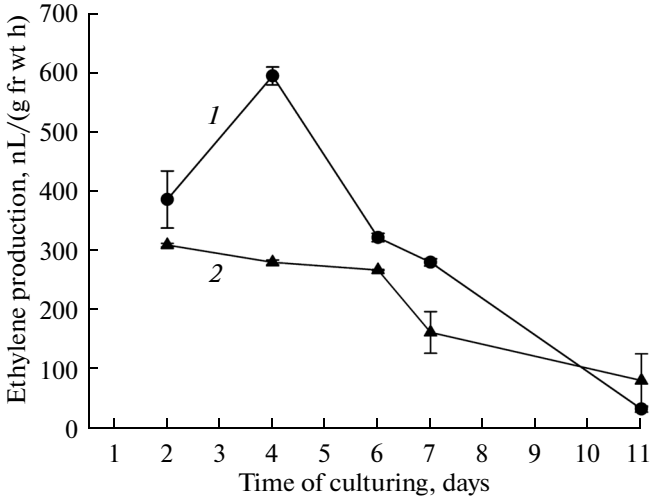

Fig. 3. The time-course of ethylene production by the Col-0 (*1*) and *etr1-1* (*2*) suspension culture cells during subculturing.

Growth curves had the form characteristic of many cultivated cells (Fig. 2). The Col-0 cell population resumed growth immediately after transfer to fresh medium and was in the logarithmic phase until the 7th day of subculturing (Fig. 2a, curve *1*); thereafter, the stationary phase started. Similar pattern was observed for cell fresh weight (Fig. 2b, curve *1*). The values of the maximal specific growth rates were close: for the number of cells μ_{max} = 0.45/day, for fresh weight – μ_{max} = 0.37/day. Growth of *etr1-1* cell population started after a lag-period (Figs. 2a, 2b, curves *2*) and continued at a lower specific rate: for the number of cells μ_{max} = 0.23/day, for fresh weight – μ_{max} = 0.29/day. It is significant that, during the entire period of subculturing, the number of cells per fresh weight in *etr1-1* was 3–4-fold higher (Table 2), which is determined by the smaller size of mutant cells.

As it was mentioned above, when cells and tissues are grown in vitro, ethylene accumulates in flasks and its concentration depends on plant species, flask sealing density, the rate of ethylene production, and conditions of cultivation [13]. Suspension cultures of Col-0 and *etr1-1* cells produced ethylene actively, and the rate of ethylene evolution decreased gradually (Fig. 3). The higher values corresponded to the logarithmic growth phase of cultures. In general, the *etr1-1* cells evolved ethylene with the lower intensity. On the second day after inoculation into fresh nutrient medium of the cells of both genotypes, flask gas contained on average 10 μL/L of ethylene. By the 7th day of culturing, the content of ethylene increased in Col-0 up to 28 μL/L, whereas in *etr1-1* it remained unchanged. These values correspond well to the measured rates of ethylene production and cell biomass in the flasks in the same time period.

It is known that the cells of essentially all plant tissues, including those of *Arabidopsis*, are mixoploid [21]. Mixoploidy is also common for in vitro cultured cells; it can be determined by both specific conditions of culturing (components of nutrient medium, temperature, etc.) and ploidy of original explants. Thus, it was shown that ethylene stimulated endoreduplication in the cells of cucumber hypocotyl epidermis; *Arabidopsis* Col-0 seedling growing in the presence of ethylene precursor, 1-aminocyclopropane-1-carbonic acid, increased the portion of polyploid nuclei in hypocotyls [22, 23]. In this connection, we analyzed the effect of ethylene produced by the cells and accumulating in the cultural flasks on nuclear DNA endoreduplication.

The cytophotometrical analysis of the nuclear DNA content in the cells of Col-0 and *etr1-1* suspension cultures was performed in two and six years of their culturing.

Figures 4a and 4b show that Col-0 culture is mixoploid. The amount of DNA in the nuclei of "young" suspension varied from 2C to 32C with the predominance of 8C (Fig. 4a). During further in vitro culturing, the portion of 4C nuclei increased and that of 8C

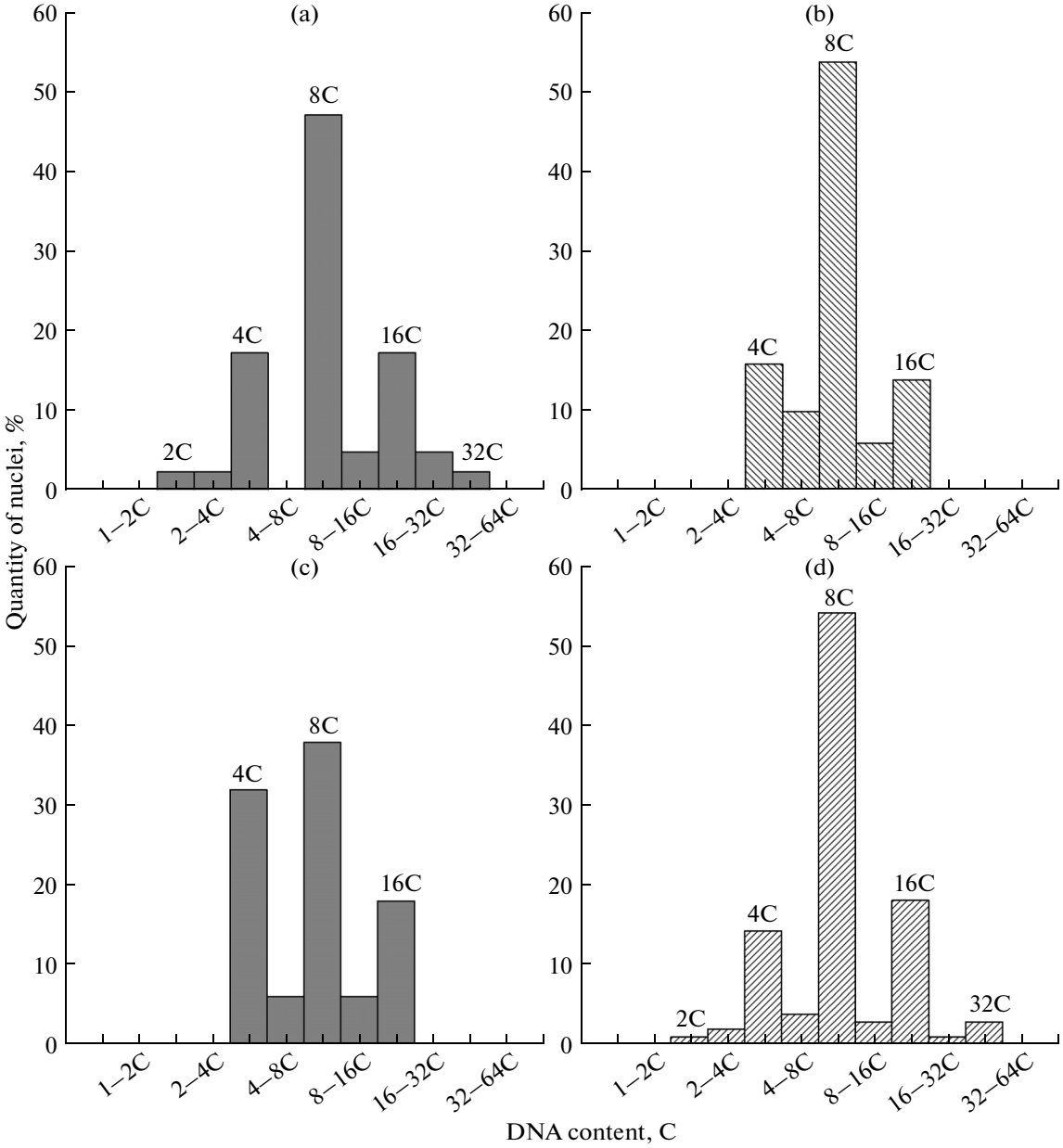

Fig. 4. Distribution of nuclei on ploidy classes in the *A. thaliana* Col-0 (a, c) and *etr1-1* (b, d) suspension culture cells. Cytophotometric analysis of the content of nuclear DNA was performed in two (a, b) and six (c, d) years after the initiation of suspension cultures.

nuclei reduced (Fig. 4c). Taking into account that our data are momentary observations for subculturings separated by a great time intervals, we can confidently speak about the stability of nuclei distribution in the DNA content in the cells of Col-0 suspension. In addition, since, for example, in *A. thaliana* Col-0 hypocotyls the ratio of cells with the amount of nuclear DNA of 2C : 4C : 8C : 16C : 32C was equal to 22 : 51 : 27 : 0 : 0 [23], the cells of the suspension Col-0 culture followed direction on substantial reduction in 2C and 4C nuclei and increase in 8C and 16C nuclei.

Distribution of nuclei on the amount of DNA in the "young" *etr1-1* cell culture (Fig. 4b) was similar to that in the Col-0 cells, but the ploidy range was narrower (from 4C to 16C) and the number of 8C nuclei was higher (54%). In vitro culturing of *etr1-1* cells for six years resulted in a insignificant widening of the ploidy range; the small portion of 32C nuclei appeared (Fig. 4d). However, the *etr1-1* cells had a pronounced model class of 8C nuclei, and this pattern persisted at long-term culturing (Figs. 4b, 4d).

Thus, the results presented showed that cultured *A. thaliana* cells genetically differing in the functional

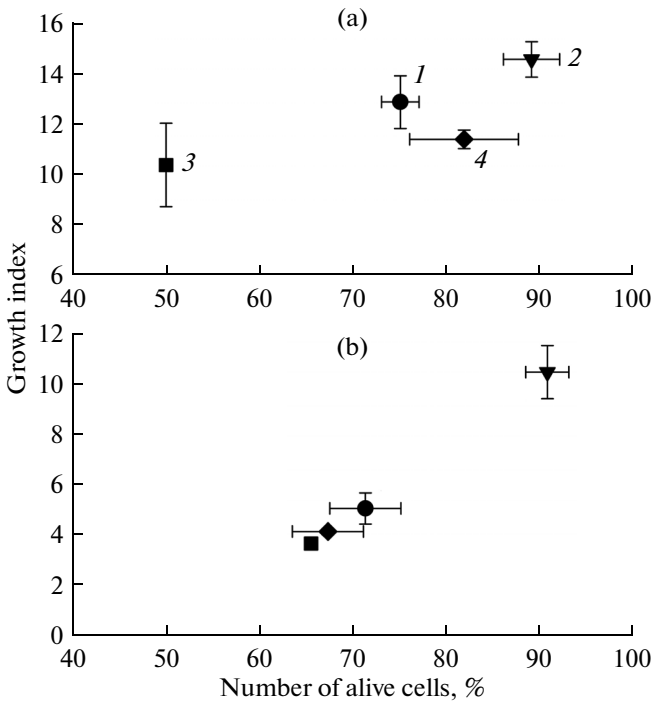

Fig. 5. Viability and growth indices of *A. thaliana* Col-0 (a) and *etr1-1* (b) suspension culture cells after cultivation in gas bells containing air (*1*), 20 μL/L of ethylene (*2*), 100 nL/L of 1-MCP (*3*), the mixture of 20 μL/L of ethylene and 100 nL/L of 1-MCP (*4*).

activity of the ETR1 ethylene receptor had some specific cytophysiological properties. First, at the disturbance of ethylene signal perception by the ETR1 receptor, the rate of in vitro cell growth declined markedly. Second, the sizes of cultured mutant cells were substantially smaller than those of Col-0 cells. Third, long in vitro cultured *etr1-1* cells preserved a capability of TE formation. Fourth, *etr1-1* cells had a pronounced model class of 8C nuclei with a tendency to widening the ploidy range toward 32C.

Nevertheless, *etr1-1* cells are already long kept in the in vitro culture. Therefore, in the next experiments we attempted to elucidate, which can be the physiological response to additionally applied ethylene on other functionally active receptors and what will be the effect of their blocking.

In investigations of the ethylene role in plant physiological processes, the inhibitors of ethylene binding with its receptors are often used along with ethylene-insensitive mutants. In our work, 1-MCP was chosen as such an inhibitor, because it is known that 1-MCP at the concentrations of 50–100 nL/L blocks ethylene action at the phytohormone concentration up to 20 μL/L [19]. We used 1-MCP at the concentration of 100 nL/L.

In preliminary experiments, we determined the composition of the gas in ~20-L gas bells after growing a standard inoculate in 8 flasks for three days. In the bell with air: O_2 – 18.5%; CO_2 – 1.75%; C_2H_4 – 1.39 ppm. In the bell with 1-MCP: O_2 – 18.5%; CO_2 – 1.82%; C_2H_4 – 1.25 ppm. It is clear that ethylene released by the cells freely went out of the flasks through the chromatography paper in the total volume of the bell. In this connection, we used the ethylene concentration of 20 μL/L similar to its usual concentration in flasks covered with foil.

The viability of Col-0 and *etr1-1* cells reduced markedly under the influence of 1-MCP on the 4th day (Fig. 5). As distinct from 1-MCP, ethylene improved the viability of Col-0 and *etr1-1* cells. Ethylene and 1-MCP affected in a similar manner on growth indices. The effect of the mixture of 1-MCP and ethylene on cell viability was intermediate.

To reveal nuclei in the phase of DNA synthesis, we analyzed the incorporation in DNA of the thymidine analog, 5-ethynyl-2'-deoxyuridine, with subsequent fluorescence detection.

In the Col-0 culture, we did not notice substantial changes in the number of S-phase cells under all experimental conditions in the beginning and at the end of the passage (Fig. 6a). In the presence of 1-MCP some S-phase cells were detected in the end of the passage; in this case growth indices were reduced (Fig. 6c).

In the culture of *etr1-1* cells, ethylene increased markedly the number of S-phase cells in the beginning of the passage (Fig. 6b). Similar pattern was observed also in the end of the passage. The inhibitor of ethylene binding with its receptors (1-MCP) exerted an opposite action. The data about the content of S-phase cells in all treatments corresponded well to growth indices of *etr1-1* culture (Fig. 6d).

The *etr1-1* cell treatment with 1-MCP alone or in combination with ethylene increased substantially the number of TE in the culture (Fig. 7); 1-MCP effect was most pronounced in the end of subculturing.

DISCUSSION

It is generally accepted that ethylene inhibits cell expansion. However, there are numerous findings that ethylene stimulates growth of internodes, leaf petioles of many plant species under flooding conditions, and hypocotyls in the light [1, 23–25]. The effect of ethylene on plant growth greatly depends on the phytohormone concentration, plant species, and growing conditions [24]. Ethylene involvement in the control of cell division is also ambiguous: a decrease in the rate of nuclear DNA replication and, as a consequence, the retardation of the mitotic cycle of pea seedlings [26], stimulation of poplar cambial cell division [27], stimulation of endoreduplication and coordinated increase of cell and organ sizes [22, 23].

Plant cells cultured in vitro are as a rule subjected to the action of high ethylene concentrations, which on the one hand favor callus formation and its subsequent growth and on the other hand can exert weak

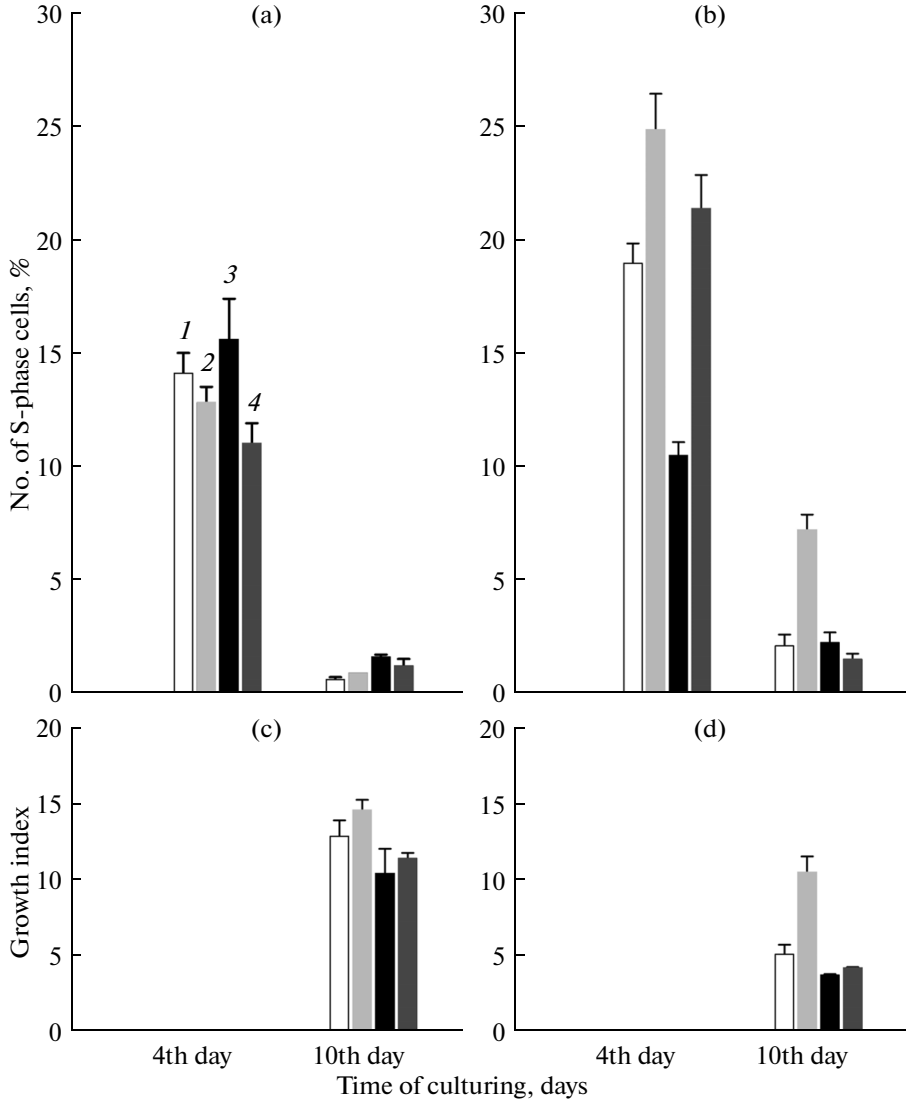

Fig. 6. Changes in the number of S-phase cells (a, b) and growth indices (c, d) of Col-0 (a, c) and *etr1-1* (b, d) cultivated *A. thaliana* cells on the 4th and 10th days of culturing in the gas bells containing air (*1*), 20 μL/L of ethylene (*2*), 100 nL/L of 1-MCP (*3*), the mixture of 20 μL/L of ethylene and 100 nL/L of 1-MCP (*4*).

negative action on the growth of suspension culture cells or confer cell insensitivity to ethylene [13].

The results presented demonstrate that suspension cultures of Col-0 and *etr1-1* cells, genetically differing by a point replacement of a single base in the triplet encoding Cys65 in the ethylene-binding site of the ethylene ETR1 receptor and grown under similar conditions, manifested substantial cytophysiological differences. The fact that cultured *etr1-1* cells are able for a long time to maintain the growth and proliferation in vitro, is hardly an argument in favor of the absence of ethylene influence on these processes.

The *etr1-1* cell population was significantly behind in growth characteristics, both the number of cells and their fresh weight (Fig. 2); the sizes of these cells were significantly less than those of Col-0 cells (Fig. 1, Tables 1, 2). The content of water in *etr1-1* cells was 88–89%, whereas in Col-0 cells – 92–93%. All this indicates a disturbance in the mutant cell expansion. In addition, cytophotometry showed a significant trend toward nuclear DNA endopolyploidization in the cells of both genotypes and the similarity of the distribution of nuclei between ploidy classes (Fig. 4). The cell sizes in the same tissue are as a rule correlated with the content of nuclear DNA [21, 22, 28], i.e., cultivated *etr1-1* cells did not grow to "permitted" sizes.

What prevents optimal cell expansion? The only obvious reason is related to the disturbance in ethylene binding with the ETR1 receptor and thus in the triggering ethylene signal transduction. It might be that under conditions of suspension culture, the cell experiences a kind of permanent "flooding," e.g., increased ethylene concentration and hypoxia, and this triggers ethylene-dependent cell expansion [24,

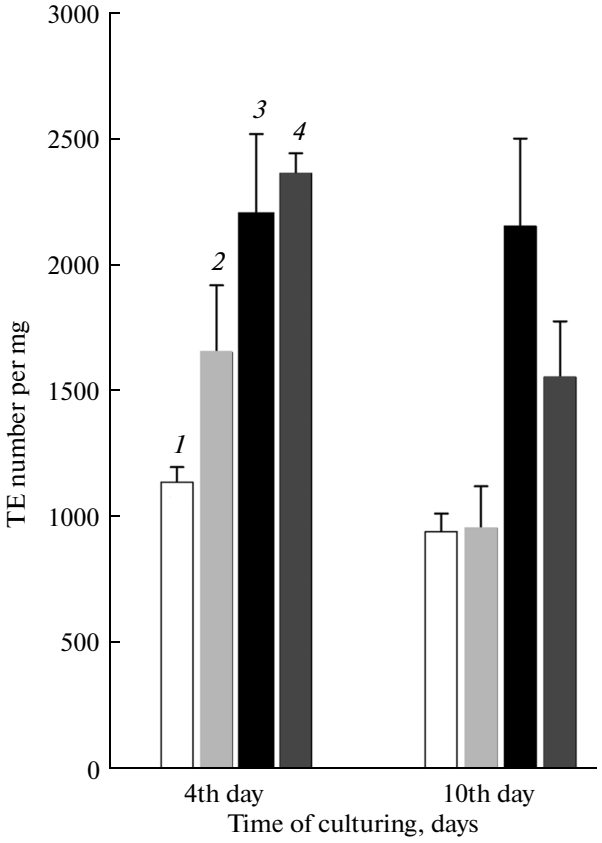

Fig. 7. Changes in the number of tracheary elements (TE) in the *etr1-1* suspension culture on the 4th and 10th days of culturing in the gas bells containing air (*1*) 20 μL/L of ethylene (*2*), 100 nL/L of 1-MCP (*3*), the mixture of 20 μL/L of ethylene and 100 nL/L of 1-MCP (*4*).

25, 29], which evidently does not function correctly in the cultured *etr1-1* cells.

Nevertheless, *etr1-1* cells respond to the additional ethylene load and 1-MCP-induced blocking of ethylene binding to the receptors. The inhibitor substantially reduced and ethylene improved the viability of both genotype cells with the same effects on growth indices (Fig. 5). Thus, ethylene signal perception and transduction are required for the maintenance of cell viability and active growth; under conditions when ETR1 receptor was inactivated, this may indicate that other receptors were responsible for these functions. In the Col-0 cell culture, there were no substantial changes in the number of S-phase cells under all experimental conditions (Fig. 6). However, in the presence of 1-MCP the greater number of S-phase cells was observed in the end of the passage at a decrease in the growth indices. It might be that 1-MCP retarded the population growth due to the lengthening of the S-phase of the cell cycle. Ethylene increased substantially the number of S-phase cells in the *etr1-1* culture, which was correlated with the growth index, whereas 1-MCP exerted an opposite action. This indicates once more that not ETR1 but other ethylene receptors are rather responsible for the control of cell proliferation. We note that at the suppression of proliferation by 1-MCP cytodifferentiation was activated, which was reflected in a substantial increase in the number of TE in the *etr1-1* culture (Fig. 7).

It is actively discussed in the literature that ethylene receptors may have non-overlapping individual properties necessary for the occurrence of a particular response to a plant hormone [30]. Further studies show, which receptor is responsible for the initiation of the signaling pathway leading to the stimulation of cell proliferation by ethylene.

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