Differences in the Manifestation of Cell Pluripotence In Vivo and In Vitro in the Mutant Arabidopsis thaliana with the Phenotype of Cell Memory Disorder

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Abstract—Plant cells cultivated in vitro are a convenient model for studying the genetic and physiological mechanisms necessary for the cells to acquire a state of pluripotency. Earlier studies on a model plant Arabidopsis thaliana (L.) Heynh. have identified the key role of genes that determine the pluripotency of cells in the shoot apical meristem in de novo shoot regeneration in tissue culture. In accordance with this, cells of mutant plants with a higher level of expression of pluripotency genes were characterized by an increased potential for de novo shoot regeneration. The tae mutant was the exception to this rule. The mutant resumed the expression of pluripotency genes and cell proliferation at the late stages of leaf development, which indicates a violation of the mechanisms for maintaining epigenetic cellular memory. At the same time, leaf cells cultured in vitro showed a lower proliferative activity compared to the wild type and were not capable of de novo regeneration of shoots. A decrease in the regenerative potential of cultured cells of the tae mutant indicates an important role of epigenetic memory in the response of cells to exogenous hormones. Impaired epigenetic memory of leaf cells of the tae mutant and differences in their proliferative and regenerative capacities in planta and in vitro make this mutant a unique model for studying the role of epigenetic modifications in the regulation of cell pluripotency.

Keywords: leaf morphogenesis, cell pluripotency, cell culture, epigenetics

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INTRODUCTION

Plants are characterized by high developmental plasticity and the ability to reprogram cells and acquire pluripotency properties. These features allow plants to form new meristems in the course of morphogenesis and to form calli at wound, ensuring the continuity of development. In this regard, the study into the genetic and physiological regulation of the plant cells pluripotency has been a topical area of plant development biology in the last decade. Cultivated plant cells are a convenient model for studying the degree of determination of somatic cells and tissues from different organs, identifying the key factors necessary for callusogenesis and shoot regeneration. Under in vitro conditions, explant cells are capable of dedifferentiation and the acquisition of a pluripotent state, which can lead to the formation of shoot and root meristems and somatic embryoids, which reflects the main strategies of plant in vivo regeneration [1].

Key factors of callusogenesis and regeneration include the content of phytohormones (exogenous phytohormones in the nutrient medium and endogenous phytohormones in the explant cells) as well as the genetically determined level of cell sensitivity to phytohormones. Stress effects—wounding, reactive oxygen species, biotic and abiotic stresses—are no less important for the initiation of in vitro and in vivo callusogenesis. Injuries lead to disruptions in intercellular communications, which play a key role in determining cell identity in plants and also cause changes in hormonal homeostasis, affecting hormone synthesis and hormonal signaling pathways [2–4].

The role of reactive oxygen species and genes that control their metabolism in the regulation of regeneration processes has been identified [5, 6]. Common genetic components and active interaction of gene networks involved in response to wounding, oxidative stress, and exposure to exogenous hormones have been determined, which explains the similar end effects of their exposure [7, 8]. The main result of the effect of hormones, injuries, and oxidative stress is cell dedifferentiation, which is caused by the activation of several groups of genes that support the indeterminacy of meristem cells [4, 5].

The main genes supporting the indeterminate state of cells of the shoot apical meristem (genes of pluripotency or stemness) include the WUS gene and the genes of class I KNOX family: KN1/BP, KN2, KN6, and STM [9, 10]. It was found that cytokinins activate the expression of the WUS gene and KNOX genes, the
products of which, in turn, activate the expression of genes for the synthesis of cytokinin [11–13]. Expression of the WUS gene also leads to suppression of the transcription of negative regulators of the cytokinin signaling pathway [14] and a decrease in the level of auxin signal in the central zone of the shoot apical meristem, protecting cells from differentiation [15]. The expression of the WUS gene is affected by the content of reactive oxygen species: hydrogen peroxide decreases, and superoxide anion increases the level of WUS expression [5], although the mechanisms of this effect have not yet been studied.

The ability of phytohormones to activate the expression of pluripotency genes and mechanisms of reverse regulation of genes for the synthesis of hormones and genes of signaling hormonal pathways are an integral part of the maintenance of meristems during plant development. Activation of pluripotency genes under stress (primarily, with wounds) is the basis of damage repair mechanisms in plants. The influence of phytohormones and stress effects on the expression of pluripotency genes also explains the ability of exogenous hormones in in vitro culture to induce reprogramming of explant cells and induce de novo organogenesis. When the function of pluripotency genes is impaired (for example, with stm or wus mutations), the ability to regenerate shoots in vitro decreases or is completely absent [16, 17]. Conversely, the overexpression of pluripotency genes leads to an increase in the in vitro regenerative capacity [18].

Overexpression of pluripotency genes (in transgenic plants or in plants with mutations in negative regulators of pluripotency gene expression) also causes disturbances in leaf morphogenesis. Leaf cells of such plants retain pluripotency, which leads to the development of ectopic outgrowths and buds on the leaves. For example, outgrowths are formed in as1 [19, 20] and as2 [21, 22] single mutants and bop1 bop2 double mutants [25]. In all cases, ectopic expression of pluripotency genes was observed in the leaves of mutants [19–21]. Pluripotency genes cease expression in leaf cells at the early stages of leaf formation in wild-type Arabidopsis thaliana plants and in other plant species with a simple leaf. This silencing is supported by epigenetic mechanisms at all subsequent stages of leaf development, which is necessary for the development of a simple non dissected leaf [23].

We have described a mutant in which we observed ectopic division of leaf cells [24], the development of the secondary leaf edge, and the formation of buds on the upper side of the leaf [25]. These processes were observed only at the late stages of leaf development, which indicates reprogramming of the genome and the acquisition of pluripotency by leaf cells.

The aim of this study is to investigate the expression of pluripotency genes and epigenetic regulators in the leaves of mutant and wild-type Arabidopsis thaliana of different ages and to assess the capability for callusogenesis and regeneration of leaf explants in vitro.

MATERIALS AND METHODS

We used the Arabidopsis thaliana (L.) Heynh. tae mutant line from the collection of the Department of Genetics, Moscow State University, which underwent seven backcrosses to the parental Blanes-M ecotype (wild type, WT). Plants were grown under long day conditions in a growth room at three temperature regimes: 26–28°C, 22–24°C, and 18–20°C. An earlier obtained mutant line containing the CycB1;1:GUS transgene in a homozygous state was used to visualize the divisions of leaf cells. The activation of the cyclin gene promoter CycB1;1 in the transgenic CycB1;1:GUS construct was judged by the expression of the uidA β-glucuronidase (GUS) reporter gene [24]. The number of outgrowths and buds was taken into account on the developed leaves of flowering plants.

For callusogenesis, we used a medium containing salts of the MS medium, Gamborg vitamins (B5), 2% sucrose, 0.8% agar, and hormones: 0.2 mg/L BAP and 1 mg/L NAA. To induce shoot regeneration, 4-week-old calli were transplanted onto a medium of the same composition but with a different hormone content: 1 mg/L BAP and 0.05 mg/L NAA. Callus cultures and parent plants (sources of explants) were grown either at 22–24°C or at 18–20°C. Explants were obtained from plant leaves at the initial stages of inflorescence development. The experiments were carried out in duplicate. 44–50 explants of each genotype were planted. The efficiency of callusogenesis was assessed as the ratio of the number of calli to the number of explants after 4 weeks of cultivation expressed in %.

RNA was isolated from leaves of plants of different ages grown at 22–24°C. RNA from calli was isolated after 3 weeks of cultivation at 22–24°C on callusogenesis medium and on regeneration medium. In the latter case, calli without buds were selected for RNA isolation. RNA for expression analysis was isolated using the RNeasy Plant Mini Kit (Qiagen, United States) according to the manufacturer’s protocol and treated with DNase (Qiagen). The first strand of cDNA was synthesized using MMLV reverse transcriptase (Sileks, Russia).

Real-time PCR (RT-PCR) was performed on an Agilent AriaMx amplifier (Agilent Technologies, United States) with a set of reagents for RT-PCR in the presence of SYBR Green dye (Sintol, Russia). The primers were synthesized at Evrogen (Russia), and the list and sequences of primers are presented in the Appendix (Supplementary Table 1). The following program was used for amplification: 1 cycle for 3 min at 95°C; 40 cycles for 15 s at 95°C, 1 min at 62°C. The analysis of the relative content of transcripts was carried out according to the $2^{-ΔΔCt}$ method. At4g33380 and At4g34270 genes were used as reference genes.
RESULTS

Expressiveness of the Phenotype of the tae Mutant and the Time of Resuming the Leaf Cell Division Depend on Temperature

The phenotype of plants of the mutant line was characterized by high variability, which showed dependence on growing temperature. At 26–28°C, the morphology of the mutant leaves was little distinguishable from the wild type: neither a characteristic narrowing of the leaf blade nor ectopic outgrowths were identified (Figs. 1a–1e). At 22–24°C, both characteristic features were noted, although their manifestation differed in time. The narrowing of the leaf blade was noticeable already during the development of the first pair of true leaves (Figs. 1d, 1e). Even the cotyledonous leaves were occasionally narrowed. At the same time, the formation of a secondary edge, outgrowths of different morphology, and, especially, buds was observed on mature leaves of plants (Fig. 1f), particularly after the inflorescence development. The anatomy of mature leaves forming outgrowths [25], as well as the features of the proliferative activity of cells in mature leaves [24], were studied in detail earlier.

Growing plants at 18–20°C caused not only narrowing but often deformation of the leaf blade (Figs. 1g–1i). A bunch of numerous underdeveloped leaves was formed in some plants (~15%) after the formation of several true leaves (Figs. 1h, 1j, 1k). Such plants did not form an inflorescence. Outgrowths and buds developed on relatively normally developed leaves (Fig. 1l). The average number of outgrowths and buds per leaf (2.5 and 0.6, respectively) was significantly higher than in plants grown at 22–24°C (1.0 and 0.14, respectively). Numerous buds could also be found in the axils of underdeveloped leaves (Fig. 1l).

To study the proliferative activity of leaf cells, we used the β-glucuronidase (GUS) reporter gene under the control of the CycB1;1 cyclin gene promoter, which allows visualization of cells at the G2/M stages. Active expression of the CycB1;1:GUS transgene in the leaves of tae plants at 18–20°C (as in the mutant at 22–24°C and in wild-type A. thaliana plants at all temperatures) could be detected at the earliest stages of leaf primordia development. As the leaf grew, the WT and the mutant cells stopped divisions, first in the distal and then in the proximal part (Figs. 2a–2c), which corresponds to the results of earlier studies into cell proliferation during the development of a simple leaf of A. thaliana [26].

At 22–24°C, cell divisions in leaves longer than 1 mm were not detected in either WT or in the mutant (Figs. 2d, 2e) and were renewed in the mutant only in mature leaves [24]. At 18–20°C, this renewal could be seen much earlier: in 5–10-mm-long leaves in juvenile plants of the mutant (Fig. 2f). Moreover, some young leaves of the upper tiers retained CycB1;1:GUS expression in the proximal and central parts for much longer (Fig. 2f, inset). Apparently, the anomalies in the development of the upper leaves of the rosette at 18–20°C (Figs. 1h, 1j–1l) are associated precisely with the presence of ectopic cell divisions in leaf primordia. The highest level of CycB1;1:GUS expression in the leaves of the mutant was observed both shortly before and after flowering (Figs. 2g, 2h) and at 22–24°C [24]. The divisions were concentrated mainly in the area of the central and lateral veins. Local accumulations of dividing cells were also detected on the periphery of the leaf blades, which led to the formation of ectopic outgrowths (Figs. 2g, 2h).

Thus, ectopic cell division in the leaves of the mutant began at 18–20°C earlier than at a higher temperature [24]. As a result, the total number of outgrowths and buds on the leaves of flowering plants turned out to be higher than that of plants grown at 22–24°C. Pluripotency genes become silent at the early stages of leaf primordium development during the development of a simple leaf in A. thaliana, and this silencing is maintained by epigenetic mechanisms throughout the ontogenesis [23]. The resumption of cell division in the mutant leaf, tested by the expression of CycB1;1:GUS and later by the appearance of outgrowths and buds on the leaf, indicates a violation of the stability of the epigenetic silencing of pluripotency genes and their reactivation in a leaf of the tae mutant.

Genes Supporting Cell Indeterminacy Resume Expression at Later Stages of Leaf Development in the tae Mutant

The expression of the main genes supporting the pluripotency of the shoot meristem cells and genes involved in maintaining the stability of epigenetic gene silencing was analyzed to test the hypothesis about the reactivation of pluripotency genes and the disturbance of the stability of epigenetic modifications. The expression analysis of the WUS, STM, KN1, KN2, and KN6 genes was carried out in the leaves of different ages in mutant and WT plants grown at 22–24°C. We used leaves without buds of four ages: 1a—leaves 2–3 mm long from 7-day-old seedlings; 2a—leaves 5–6 mm from 14-day-old juvenile plants; 3a—17–22 mm leaves from 21-day-old plants; 4a—mature leaves from flowering plants.

We failed to reveal the expression of the studied pluripotency genes in 1a and 2a young leaves in either the WT or the mutant. Apparently, the expression of these genes is suppressed at the early stages of leaf development both in the mutant and in the WT plants. The mutant had an increased expression of the KN1 gene in 3a leaves (Fig. 3a). The expression level of other genes did not differ from the control (WT leaves of the same age). The mutant showed an increase in the expression of all genes in 4a mature leaves, except for the WUS gene, while WT showed only trace amounts of KN1 and KN2, and the expression of the KN6 and STM genes was not detected (Fig. 3a, Supplementary Fig. 1). The time of reactivation of pluripotency genes coincides with the time when cell division and expression of CycB1;1:GUS are resumed in
Fig. 1. Temperature dependence of the tae mutant phenotype. The upper, middle, and two lower lines are plants under conditions of 26–28°C, 22–24°C, and 18–20°C, respectively. (a, b) 3-week-old WT and tae plants, respectively; (c) 4-week-old WT (left) and tae (right) plants; (d, e) 3-week-old WT and tae plants, respectively; (f) 4-week-old WT (left) and tae (right) plants; (g, h) WT and tae plants at the age of 1.5 months, respectively (the upper leaves of the tae rosette stopped growing; (c) cotyledons); (i) deformed leaves of 2-month-old mutant plants with outgrowths and buds; (j–l) scanning electron microscopy of the leaves of tae plants shown in h; (j) a bunch of underdeveloped leaves at the top of the shoot; (k) leaves that have stopped developing at different stages of development; (l) base of one of the apical leaves with numerous buds. Bars correspond to (a–i) 1 cm, (j, k) 300 μm, (l) 100 μm.
leaves. These data indicate that the activation of divisions is the result of the resumption of expression of pluripotency genes in the leaves of the mutant.

**Genes Controlling DNA Methylation and Histone Modification Altered Expression in a Leaf of the tae Mutant**

The expression of epigenetic regulators was studied in the leaves of ages 2a, 3a, and 4a (mature leaf). We analyzed the expression level of three genes that control DNA methylation: *MET1* (methylation of CG-sites), *CMT3* (methylation of CXG-sites), and *DDM1* (chromatin-mediated methylation of DNA and H3 histone), two genes of histone deacetylases (*HDA13* and *HDA17*), *SUVR2* gene involved in RNA-dependent DNA methylation, and the *SUVH5* gene, which encodes a histone methyltransferase that maintains the H3mK9 mark and is involved in *CMT3*-dependent
DIFFERENCES IN THE MANIFESTATION OF CELL PLURIPOTENCE

The expression levels of $HDA13$ and $HDA17$ genes encoding the histone deacetylases were lower in the mutant compared to the WT in 3a and 4a leaves, although the level of $HDA13$ in 2a leaves was higher than in WT. A similar dynamics of a decrease in the expression level with increasing age was also found for the $SUVR2$ gene. The mutant showed a transient increase in the expression of the $SUVH5$ gene in 3a leaves, although the expression level of $SUVH5$ in mature leaves (4a) was lower than in the WT leaves of the same age (Fig. 3b). Although the expression analysis of individual representatives from different groups of epigenetic regulators does not give a complete picture of the processes occurring in the genome, the changes in their expression found in the leaves of the mutant confirm the assumption about the processes of reprogramming of the genome, which are necessary for the reactivation of pluripotency genes.

Cells from the Leaves of the tae Mutant Show no Pluripotency in vitro

Explants of mature leaves were used to study the manifestation of cell pluripotency in vitro. Callusogenesis from explants of the tae mutant turned out to be less effective compared to the WT (Table 1). The growth rate of mutant calli was also significantly lower than that of WT calli. The average mass of callus from mutant leaves at the end of the month of cultivation at a temperature of 18–20°C was two times lower than that of WT calli (Table 1). At 22–24°C, calli grew better, but the differences between the tae mutant and WT remained. The growth retardation of calli from the mutant persisted in the regeneration medium (Figs. 4a, 4b).

The expression of the $CycB1;1:GUS$ transgene in the calli of the mutant was also lower than in the calli of WT (Figs. 4c–4f). Thus, ectopic divisions, which are characteristic of leaves, were not detected in calli from the same leaves. Unlike calli from WT leaves, calli from the mutant were not able to regenerate shoots either. This ability did not depend on temperature (Table 1). The proportion of calli with shoots from WT leaves at 18–20°C was significantly lower than at 22–24°C (Table 1). Thus, mutant cells showed a reduced proliferation rate in vitro, did not show the ability to form buds even under temperature conditions that enhance ectopic cell proliferation in the leaf in vivo.

Table 1. Comparison of callusogenesis and the ability to regenerate shoots in tissue culture from leaves of tae mutant and WT

| Conditions | Genotype | Efficiency of callusogenesis, % | Callus mass, mg | Calli with buds, % | Number of buds/callus |
|------------|----------|---------------------------------|----------------|-------------------|----------------------|
| 18–20°C    | WT       | 100                             | 540 ± 45       | 6.8 ± 0.8         | 0.1 ± 0.1            |
|            | tae      | 81.5 ± 7.0                      | 225 ± 24       | 0                 | 0                    |
| 22–24°C    | WT       | 100                             | 600 ± 60       | 42.0 ± 5.0        | 0.8 ± 0.1            |
|            | tae      | 86.0 ± 8.1                      | 450 ± 45       | 0                 | 0                    |
Analysis of the expression of pluripotency genes on the callusogenesis medium and on the regeneration medium showed an increase in the expression of only one gene, *KN2*, in the mutant compared to the WT (Fig. 5). The expression of the *WUS* and *STM* genes was lower than in the calli of the WT, and the expression level of the *KN1* gene either did not differ from the WT (on the callusogenesis medium) or was lower (on the regeneration medium).

Thus, mutant cells did not exhibit any ectopic divisions in tissue culture and increased expression of most genes of pluripotency, which is characteristic of mature leaf cells. These features of mutant cells did not depend on the content of exogenous hormones,
Differences in the Manifestation of Cell Pluripotence

Proliferation of leaf primordia cells in seedlings of the tae mutant had the same regularities as in the WT. Expression of the marker of cell divisions (CycB1;1:GUS transgene) was first observed in the entire leaf primordium and then ceased: first in the distal part of the leaf primordium and then in the proximal part (Figs. 2a–2c). No divisions were detected in leaves larger than 1 mm. Further growth of the leaf was ensured by cell enlargement, which is characteristic of the simple leaf of Arabidopsis thaliana. Loss of proliferation by leaf cells is the result of transcriptional repression of pluripotency genes at early stages of its development. Silence of pluripotency genes is maintained by epigenetic mechanisms at all subsequent stages of ontogenesis, causing the development of a simple undissected leaf [23]. The main differences include the resumption of cell proliferation and expression of pluripotency genes at the late stages of leaf ontogenesis, which indicates impairments in the mutant’s epigenetic cellular memory. These features of leaf morphogenesis, together with the lack of regenerative abilities in the cells of the tae mutant in vitro, make it a unique model for studying the role of epigenetic modifications in the regulation of cell pluripotency.

The instability of epigenetic changes can lead to reprogramming of the genome and activation of silent pluripotency genes. The presence of such reprogramming is indicated by the change found in the expression of a number of epigenetic regulators in leaves of the mutant of different ages (Fig. 3b), reactivation of pluripotency genes (Fig. 3a), and resumption of cell divisions with the growth of the leaf (Fig. 2). Genetically determined changes in epigenetic regulators in the tae mutant seem to influence the processes of callusogenesis and in vitro regeneration.

The role of epigenetic processes in the regulation of in vitro callusogenesis and in vitro regeneration is evidenced by the obtained results of the transcriptomic analysis and the dynamics of changes in DNA methylation and modification of histone proteins [27]. The role of epigenetic modifications in the regulation of differentiation of explant cells, callusogenesis, and regeneration is also evidenced by serious disturbances of these processes by mutations in genes that carry out DNA methylation and histone modification [10, 28, 29].

Epigenetic modifications can affect the processes of callusogenesis and regeneration by altering the expression of many genes that control hormonal homeostasis: hormone metabolism and hormonal response. The epigenetic regulation of the expression of genes for the synthesis, catabolism, and transport of auxin, and genes of the auxin response, has been studied most fully [30]. It cannot be ruled out that the previously identified changes in auxin homeostasis in the tae line [24] are also associated with changes in the functioning of epigenetic regulators. Epigenetic changes may also underlie the insensitivity of cultured mutant cells to exogenous hormones, which affects the efficiency of callusogenesis and regeneration of leaf explants in vitro.

The tae mutant has unique characteristics of the anatomical structure and ontogeny of the leaf [24, 25], which distinguish it from earlier-studied mutants and transgenic plants with ectopic expression of pluripotency genes in the leaf. The main differences include the resumption of cell proliferation and expression of pluripotency genes at the late stages of leaf ontogenesis, which indicates impairments in the mutant’s epigenetic cellular memory. These features of leaf morphogenesis, together with the lack of regenerative abilities in the cells of the tae mutant in vitro, make it a unique model for studying the role of epigenetic modifications in the regulation of cell pluripotency.

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Compliance with ethical standards

Conflict of interests. The authors declare that they have no conflicts of interest.

Statement on the welfare of humans or animals. This article does not contain any studies involving animals performed by any of the authors.
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SUPPLEMENTARY MATERIALS
Supplementary materials are available for this article at https://doi.org/10.1134/S1021443721010106 for authorized users.

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RUSSIAN JOURNAL OF PLANT PHYSIOLOGY Vol. 68 No. 1 2021
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