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Original Study Article

Analysis of the role of tonoplast H^+-ATPase in elongation growth of coleoptile cells of rice seedlings with different growth rates under normoxia and submergence

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ABSTRACT

BACKGROUND: Rice coleoptiles were used to investigate the importance of V H^+-ATPase in vacuolization during elongation growth under normoxic and hypoxic conditions.

AIM of the study was to find out a link between growth intensity, protein amount of subunits B and E and transcription of genes encoding those proteins.

MATERIALS AND METHODS: The investigation was carried out on two rice varieties of domestic selection, fast-growing Kuban 3 and slow-growing Amethyst. Seedlings were grown in etiolated conditions at normoxia and submergence. Western-blot analysis was employed to evaluate amount of subunits B and E in microsomal fraction. qRT-PCR was used to distinguish differences in expression of genes encoding subunits B and E of V H^+-ATPase.

RESULTS: The growth under aerobic conditions was more consistent with the changes in subunits B and E of V H^+-ATPase which was determined at the proteomic level, while the hypoxic growth had a stronger correspondence with changes in *OsVHAs* gene expression. Varietal differences were revealed only when comparing the transcription intensity, which did not affect the growth dynamics of coleoptiles. Obtained data suggested the existence of differences in the regulation of the enzyme at the transcriptional and proteomic levels during coleoptile elongation.

CONCLUSIONS: The importance of the B and E subunits of V-ATPase involvement in vacuolization during the growth process of rice coleoptiles under different oxygen level was demonstrated.

Keywords: hypoxia; rice *Oryza sativa* L.; vacuolar H^+-ATPase.

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Оригинальное исследование

Анализ роли H^+-АТФазы тонопласта в обеспечении роста растяжением клеток колеоптилей проростков риса, различающихся скоростью роста в условиях нормоксии и затопления

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АННОТАЦИЯ

Актуальность. Одна из стратегий адаптации к затоплению у растительных организмов заключается в усилении роста с целью избежать повреждающего воздействия недостатка кислорода. К модельным объектам для изучения данного типа роста относят колеоптили проростков риса, обладающего способностью к прорастанию под водой. Длина колеоптилей служит маркером жизнеспособности при гипоксии.

Цель — сравнительный анализ роста колеоптилей и участия субъединиц B и E вакуолярной H^+-АТФазы в его реализации у двух сортов риса отечественной селекции, различающихся по исходной скорости удлинения (быстро растущий Кубань 3 и медленно растущий Аметист).

Материалы и методы. Исследование проведено на этилированных проростках колеоптилей риса двух сортов отечественной селекции Кубань 3 и Аметист. Детекцию субъединиц B и E V-АТФазы в составе общей микросомальной фракции реализовывали с использованием иммуно-блотт-анализа. Интенсивность транскрипции генов, кодирующих субъединицы B и E V-АТФазы, определяли методом полимеразной цепной реакции с обратной транскрипцией.

Результаты. Динамика роста в условиях аэрации более соответствовала изменениям субъединиц, происходящих на протеомном уровне, тогда как динамика роста при затоплении имела большее сходство с изменением транскрипции генов, кодирующих эти субъединицы. Сортовые различия были выявлены только при сравнении интенсивности транскрипции, что в конечном итоге не сказывалось на динамике роста колеоптилей.

Выводы. Показано участие субъединиц B и E V-АТФазы в обеспечении вакуолизации в процессе роста растяжением колеоптилей риса при различном содержании кислорода.

Ключевые слова: гипоксия; рис *Oryza sativa* L.; вакуолярная H^+-АТФаза.

Как цитировать

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BACKGROUND

The rapid growth potential in plants can be considered as an alternative to behavioral flexibility in animals aimed at avoiding damage from external stressors. In plant organisms that practice a sedentary lifestyle, the low-oxygen escape syndrome (LOES) is one of the strategies for adaptation to flooding [1]. This type of growth response can be seen in various aquatic plants [2]. These include Asian cultivated rice (*Oryza sativa* L.), a valuable crop that can germinate and grow rapidly under flooding conditions, thus overcoming oxygen deficiency. Germination is associated with accelerated growth of the coleoptile, a juvenile organ [3–5]. Coleoptile is a modified leaf that forms a tubular sheath in cereals and exhibits a variety of defense responses during hypogeal germination [6]. A longer coleoptile reduces or even prevents damage from stressors such as drought, sharp temperature drops (frosts), herbicides, and even rodents [7–9]. In rice seedlings, this defense response is very special. The hypothesis proposed back in the 1970s suggests that the coleoptile serves as a "snorkel" through intensified growth and the ability to quickly reach the water surface [10]. This hypothesis remains relevant [6, 11].

The growth of coleoptiles is primarily driven by plant cell-specific elongation, which involves multiple polar (vertical) elongations. This process is short-term, and its intensity is determined by the osmotic pressure vector with a sharp increase in cell wall elasticity. The mechanisms that initiate and regulate elongation formed the basis of the acid-growth hypothesis, which is still relevant today, despite being proposed almost half a century ago [12, 13]. Under normal oxygen conditions, plant hormone auxin activates the plasma membrane H^+-ATPase, acidifying cell walls and activating several cell elongation mechanisms.

Moreover, elongation is accompanied by intensive vacuolization. However, the mechanisms of this process are still poorly understood. There is no doubt about the sharp increase in water and osmotically active compound transport through across the tonoplast, or vacuolar membrane. The vacuolar H^+-ATPase (V-ATPase; EC 3.6.1.3), which is prevalent in eukaryotic cells, plays the defining role in membrane potential generation on the tonoplast [14, 15]. This proton-transporting multi-subunit enzyme is known to be involved in plant adaptation to various stress factors and has even been termed the *eco-enzyme* [16]. The composition of isoforms that comprise the vacuolar H^+-ATPase structure is tissue-specific [16, 17]. However, the role of this pump in growth, especially under oxygen deficiency, remains uncertain. Changes may be related to regulation at both the transcriptional and post-translational levels.

The study aimed at comparing the growth intensity, expression of genes encoding subunits B and E, and their abundance in coleoptile cell membranes of rice seedlings of two varieties differing in growth intensity under normoxic and flooding conditions.

METHODS

Study Objects

Coleoptiles of 3-, 5-, and 7-day-old rice (*Oryza sativa* L.) seedlings were used in the experiments. These time points correspond to the beginning, intensification, and completion of coleoptile growth, respectively. They were selected during the preliminary stage of the study. The study used caryopses of two Russian rice varieties: slow-growing Amethyst and fast-growing Kuban 3 [5]. The seeds were surface sterilized with a 50% sodium hypochlorite solution for 15 min, rinsed with sterile water 10 times, and soaked in hot water (55°C) for 1 h. Next, 50 seeds were germinated under hydroponic conditions on glass bridges (control plants) or under simulated flooding conditions in 750 mL jars, as previously described [5]. A 4% Knop's solution was used [18]. The O_2 level was measured using the Expert-009 dissolved oxygen analyzer (Econix-Expert, Russia). The oxygen content in the hypoxic solution did not exceed 0.5–0.6 mg/L. The glassware and solutions were presterilized.

To measure the length of coleoptiles, the seedlings were placed on Petri dishes and scanned using the HP ScanJet G2710. The photos were then digitized in ImageJ (version 1.8.0_172) [5]. The analysis included all germinated plants from the 50 seeds.

Obtaining the Total Microsomal Fraction of Rice Coleoptile Cells

The total microsomal fraction was obtained from coleoptile cells of Amethyst and Kuban 3 seedlings at 4°C. A plant material sample (approximately 1 g) was homogenized in a medium consisting of 330 M sucrose, 50 mM Tris-HCl, 5 mM ethylenediaminetetraacetic acid, 5 mM ascorbic acid, and 5 mM dithiothreitol (pH 7.8) [19]. The resulting homogenate was centrifuged for 10 min at 100 g (MPW-350R, Poland). The speed was then gradually increased, and the homogenate was centrifuged for 5 min at 3,000 g to separate heavy cell components (nuclei, cell wall fragments), followed by 15 min at 17,000 g to precipitate mitochondria. The resulting precipitate was homogenized in a medium containing 300 mM sucrose solution in 10 mM Tris-Mes buffer (pH 7.2). The supernatant was centrifuged at 100,000 g on the Beckman Avanti J-30I centrifuge (USA) for 60 min. The precipitate (total microsomal fraction) was homogenized in a medium containing 300 mM sucrose solution in 10 mM Tris-Mes buffer (pH 7.2).

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Denaturing Polyacrylamide Gel Protein Electrophoresis

Protein separation was performed by denaturing electrophoresis in a 10% polyacrylamide gel [20]. Before applying electrophoresis gels, the samples were equalized by protein content, which was measured using the Bradford protein assay [21]. Protein from the total microsomal fraction of cell membranes was precipitated with 20% trichloroacetic acid and dissolved in a loading buffer. Twenty micrograms of protein were added to the gel. PageRuler™ Prestained Protein Ladder molecular weight markers (Thermo Fisher Scientific, USA) were used to control gel separation of proteins. Electrophoretic separation of proteins was performed in a Tris-glycine buffer (25 mM Tris, 192 mM glycine, 0.1% sodium dodecyl sulfate, pH 8.3) using the Mini-PROTEAN system (Bio-RAD, USA) at 4°C.

Tonoplast H^+-ATPase Subunit B and E Assay in the Total Microsomal Fraction of Rice Coleoptile Cells by Immunoblotting

After electrophoresis, the gels were washed with a transfer buffer. The Mini-PROTEAN system (Bio-RAD) was then used for a blot transfer onto a 0.45 μm nitrocellulose membrane (Bio-RAD) for 1 h at 100 V and 250 mA, according to the manufacturer's protocol. After the transfer, the membrane was placed in a blocking buffer for 1 h. The membrane was then incubated overnight in a blocking buffer containing primary rabbit antibodies specific to tonoplast H^+-ATPase subunits B (AS09 503) and E (AS07 213) (Agrisera, Sweden). Following that, the membrane was washed five times in 25 mL of blocking buffer for 5 min each. Secondary polyclonal goat anti-rabbit antibodies labeled with horseradish peroxidase (AS09 602, Agrisera) were dissolved in 25 mL of blocking buffer with milk. The membrane placed in a solution with secondary antibodies was kept on a shaker at 37°C for 1 h. After washing the membrane, tonoplast H^+-ATPase subunit stains on the nitrocellulose membrane were produced using 3,3-diaminobenzidine dissolved in a phosphate-buffer saline (pH 5.8). After staining, the membrane was scanned. The PhotoM software was used to measure the absorbance and area of stains, which represented the degree of interaction between tonoplast H^+-ATPase subunits and antibodies. These parameters were then multiplied and compared to the control (normoxic) values on day 3 of germination, which were set equal to one.

Primer Design and Real-Time Quantitative PCR

The annotated rice genome databases (The Rice Annotation Project, RAP, https://rapdb.dna.affrc.go.jp/) and VectorNTI Advanced v.11 were used to select primers. Primers were selected according to real-time PCR conditions, with the SYBR Green I intercalating dye (Evrogen, Russia). Primer parameters: melting temperature 58–65°C; length 20–30 bp; hairpin structure and dimer formation in a primer pair with dG> –1 kcal/mol; GC content 40%–60%. Specific primers were selected for all sequences of interest. The specificity of primers was checked using the NCBI BLASTn algorithm (https://blast.ncbi.nlm.nih.gov). The primer sequences are shown in Table 1. The primers were synthesized at BioBeagle (Russia, https://biobeagle.com/).

cDNA Synthesis

RNA was isolated from rice seedling coleoptiles (in the control group, leaves were removed because leaves do not grow inside the coleoptile under hypoxic conditions) at the respective germination time points (3, 5, and 7 days) using the ExtractRNA reagent (Evrogen, Russia), according to the manufacturer's protocol. DNase treatment was performed using DNase by Thermo Fisher Scientific, USA, 5 units/sample, according to the

Table 1. Primers for genes of interest and comparison

Таблица 1. Праймеры к генам интереса и сравнения

Gene	Locus	Primers (5'–3')	Product length, bp
OsVHA-B1	Os06t0568200	GTGAGGTATCAGCAGCCCGAGAA CCCGTAAGATCAGGAGTTGGATGTG	187
OsVHA-B2	Os01t0711000	CAATCCCAGTGAACGAACATACCCT TCTGAGCAGCAATTTCATTGTGTGG	143
OsVHA-E1	Os01t0659200	CCAAGCAGATCCAGCAGATGGTG TTGAACTCCTCCTCGGCGGA	91
OsVHA-E2	Os05t0480700	AGCAGATCCAGCAGATGGTCAGG TGATCCTCCGCTTCTCCGACTC	132
OsTUB4	Os01g0805900	GAACCATTTGATTTCTGCCACCA CGGTACTGCTGGGAGCCACG	171

manufacturer's protocol. Purified RNA was dissolved in sterile water and stored at –80 °C until analysis.

The MMLV RT kit (Evrogen, Russia) was used for reverse transcription, according to the manufacturer's protocol. cDNA was synthesized using 2 μg of RNA. The obtained cDNA was aliquoted and stored at –80°C until analysis.

Assessing the Expression of Genes Encoding Tonoplast H^+-ATPase Subunits B and E

Real-time quantitative PCR was performed using the Bio-Rad CFX96 REAL-TIME System (USA; at Saint Petersburg State University's Center for Molecular and Cell Technologies) and the 5X qPCRmix-HS SYBR detection kit (Evrogen, Russia). Amplification was performed as follows: at 95°C for 5 min; at 95°C for 15 s; at 60°C for 30 s; at 72°C for 30 s; a total of 45 cycles. The fluorescence of the SYBR Green I intercalating dye was assessed at the end of each cycle. The $2^{-\Delta Ct}$ method was used to determine the relative number of transcripts from threshold amplification cycles (Ct). The $2^{-\Delta\Delta Ct}$ method was used to assess the rate of change in the relative number of transcripts for each gene [22]. The quantitative assessment of the analyzed gene is reported in relative units, calculated by comparing the expression level to that of the β-tubulin 4 gene (*OsTub4*). Changes in expression levels of the genes of interest were calculated relative to the control (normoxic) values on day 3 of germination, which were set equal to one.

Statistical Processing

All experiments had 4–8 biological replicates and 3 analytical replicates, except for immunoblotting, which had 3 biological replicates. Statistical analysis was performed using GraphPad Prism 8.0.1 for Windows. Figs. 1–3 show the mean values and their standard errors. Values with different letters differ significantly at p <0.05 (Tukey's weighted mean).

RESULTS

Under normal oxygen conditions, the coleoptiles of Kuban 3 seedlings showed an intense onset of growth (reaching 10 mm by day 3), a twofold increase in length by day 5, and a subsequent cessation of elongation by day 7 (Fig. 1). On days 3 and 5, the coleoptiles of Amethyst seedlings showed much less elongation, averaging approximately 50% of that of Kuban 3 seedlings. Later, the growth was not compensated by greater duration and also ceased by day 7. Thus, both varieties had identical growth patterns; however, the growth amplitude in Amethyst was almost half as much. Germination under flooding conditions led to changes in the growth pattern. Notably, a significant growth inhibition was observed for Kuban 3 coleoptiles at the initial stage of germination (by day 3). Even more remarkable is the subsequent increase in elongation; as a result, by day 5, the length under hypoxic conditions was equal to that in the control group. Furthermore, the growth continued, if marginally, on day 7 under hypoxic conditions. The coleoptiles of Amethyst seedlings showed more than a twofold growth inhibition compared to normal oxygen conditions. Subsequently, from day 3 to day 5, the growth intensified and continued till day 7. Nevertheless, the length of Amethyst coleoptiles at the final time point of the experiment was almost 40% shorter.

A total microsomal membrane fraction containing the tonoplast was obtained for the subsequent analysis of the prevalence of two vacuolar H^+-ATPase subunits in coleoptile cells of rice seedlings. In this series of experiments, the protein level in isoforms of interest, measured by the degree of interaction with specific antibodies, on day 3 of development under normal oxygen conditions was set equal to one. The identified patterns are shown in Fig. 2. In the coleoptiles of both rice varieties, V-H^+-ATPase subunits B and E with a molecular mass of 55 kDa and

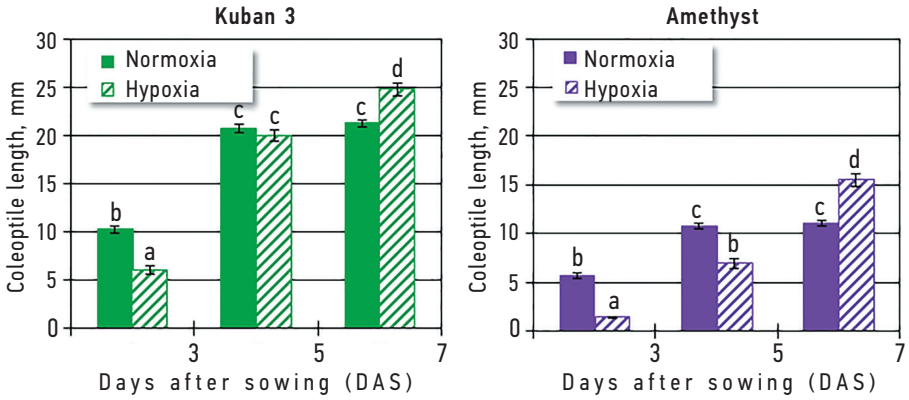

Fig 1. The growth of rice seedlings coleoptiles of two varieties (fast-growing variety Kuban 3; slow-growing variety Amethyst) under normoxia and hypoxia. Values with different letters (a–d) are significantly different (Tukey's test, p <0.05).

Рис 1. Рост колеоптилей проростков риса двух сортов (быстро растущий сорт Кубань 3; медленно растущий сорт Аметист) в условиях нормоксии и гипоксии. Значения с разными буквами (a–d) достоверно различаются (взвешенное среднее Тьюки, p <0,05).

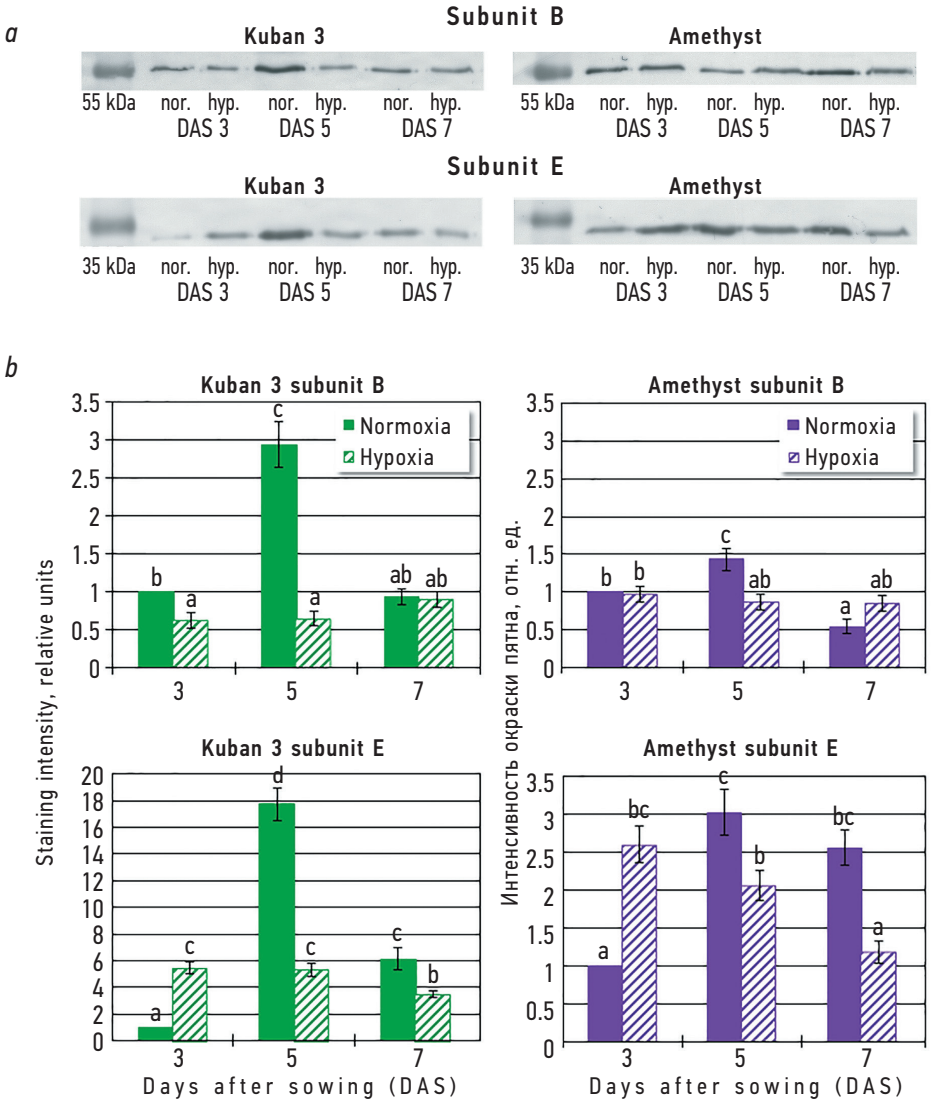

Fig 2. Alteration in the content of proteins of B and E subunits of H^+-ATPase tonoplast in the microsomal fraction of coleoptile cells of rice seedlings of two varieties (fast-growing variety Kuban 3; slow-growing variety Amethyst) under normoxia and hypoxia: *a*, western blot hybridization of microsomal fraction protein samples with antibodies against B and E subunits. 35 and 55 kDa, molecular weight markers; nor., normoxia, hyp., hypoxia; Scanned images of typical blots; *b*, relative change in protein content. Values with different letters (a–d) are significantly different (Tukey's test, p <0.05).

Рис 2. Изменение содержания белков субъединиц B и E H^+-АТФазы в составе микросомальной фракции клеток колеоптилей проростков риса двух сортов (быстро растущий сорт Кубань 3; медленно растущий сорт Аметист) в условиях нормоксии и гипоксии: *a* — вестерн-блот гибридизация проб белка микросомальной фракции с антителами против субъединиц B и E. 35 и 55 кДа — маркеры молекулярного веса; нор. — нормоксия, гип. — гипоксия. Отсканированные изображения характерных блотов; *b* — относительное изменение содержания белков. Значения с разными буквами (a–d) достоверно различаются (взвешенное среднее Тьюки, p <0,05).

approximately 31 kDa, respectively, were detected (Fig. 2, *a*). Under normal oxygen conditions, the level of subunit B sharply increased (by more than 2.5 times) by day 5 of growing Kuban 3 seedlings (Fig. 2, *b*). During subsequent development (on day 7), it returned to baseline. Comparable changes in subunit B levels were observed for the coleoptiles of Amethyst seedlings; however, the amplitude was significantly lower and was below baseline (less than one) by day 7. The changes were entirely different when growing seedlings under flooding conditions. On day 3, the level of subunit B in the membranes of Kuban 3 coleoptile cells was significantly lower than in the control group. Following that, a weak upward trend was observed, but the value never reached one. Amethyst coleoptiles likewise had a low level of subunit B protein in endomembranes, with values close to one at all time points.

A similar trend was observed for subunit E (Fig. 2). Under normal oxygen conditions, the maximum content of proteins of interest in Kuban 3 coleoptile membranes was observed on day 5 of development, which then decreased but still exceeded that on day 3. The Amethyst variety

showed similar nonlinear changes in protein levels, albeit much less pronounced. However, under flooding conditions, changes in subunit E levels differed from those under normal oxygen conditions, as well as from those for subunit B. Notably, on day 3 of development, oxygen deficiency increased the content of isoform E in coleoptile cell membranes of Kuban 3 seedlings. On day 5, the content did not change and even slightly decreased in the final time interval. A similar trend was observed for coleoptile cells of Amethyst seedlings. Thus, changes in the isoenzyme composition of vacuolar H^+-ATPase corresponded to the coleoptile growth pattern under normal oxygen conditions, but differed significantly from that under flooding conditions.

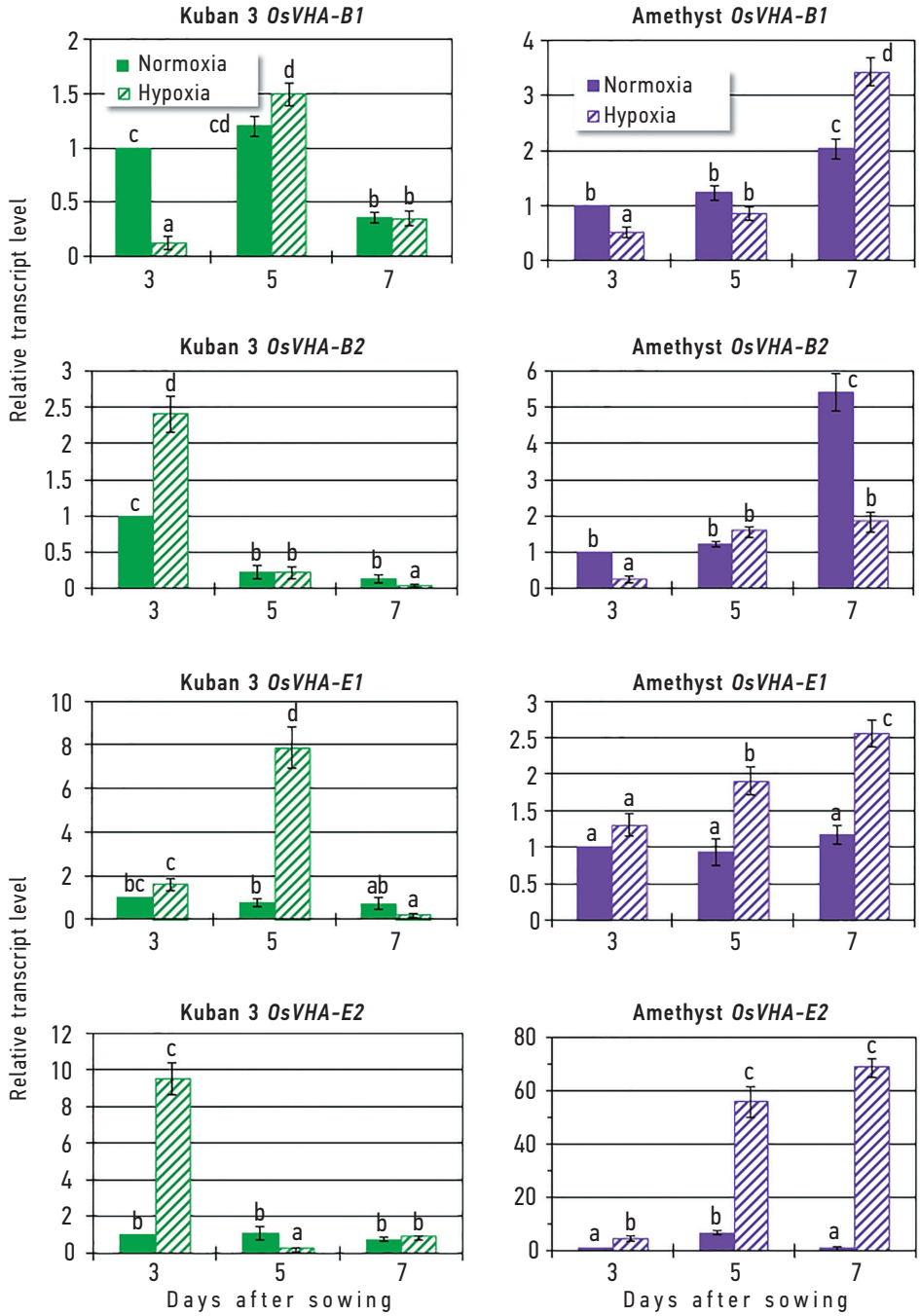

Fig 3. Changes in the relative level of accumulation of *OsVHA-B1*, *OsVHA-B2*, *OsVHA-E1*, and *OsVHA-E2* gene transcripts in coleoptiles of rice seedlings of two varieties (fast-growing variety Kuban 3; slow-growing variety Amethyst) under normoxia and hypoxia conditions. Values with different letters (a–d) are significantly different (Tukey's test, p <0.05).

Рис 3. Изменение относительного уровня накопления транскриптов генов *OsVHA-B1*, *OsVHA-B2* и *OsVHA-E1*, *OsVHA-E2* в колеоптилях проростков риса двух сортов (быстро растущий сорт Кубань 3; медленно растущий сорт Аметист) в условиях нормоксии и гипоксии. Значения с разными буквами (a–d) достоверно различаются (взвешенное среднее Тьюки, p <0,05).

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The study concluded by analyzing the expression of genes encoding subunits B and E under similar germination conditions (normoxic and hypoxic) (Fig. 3). The increase in *OsVHA-B1* gene transcripts in the Kuban 3 variety was nonlinear, peaking on day 5. Under flooding conditions, the most significant increase was observed between days 3 and 5 of development. In Amethyst coleoptile cells, the changes were completely different: transcripts accumulated gradually, and their level peaked on day 7, regardless of oxygen supply. However, the magnitude of these changes was also higher under flooding conditions. Significant differences were detected during the transcription analysis of the *OsVHA-B2* gene. Transcript accumulation reduced with age in Kuban 3 seedlings, which was more pronounced in oxygen deficiency. Amethyst seedlings showed directly opposite changes, peaking on day 7 and under normal oxygen conditions.

Regarding the *OsVHA-E-1* gene, there were no significant changes under normal oxygen conditions in both varieties. However, under hypoxic conditions, the highest transcript accumulation in coleoptile cells was reported on day 5 in Kuban 3 seedlings and on day 7 in Amethyst seedlings. Of note are changes in the expression of the *OsVHA-E2* gene. There were no changes in transcripts during the development of Kuban 3 seedlings in the control group. However, the situation changed drastically under flooding conditions: the maximum was reported on day 3, exceeding the value under normal oxygen conditions by almost 9 times. In Amethyst seedlings, there was a slight nonlinear change in transcript accumulation (peaking on day 5) under normal oxygen conditions. Under flooding conditions, transcript levels gradually increased over time, reaching a 70-fold increase in 7-day-old seedlings. The study findings indicate that the observed changes in the expression of genes encoding vacuolar H^+-ATPase subunit B and E isoforms differed from the results of immunoblotting.

DISCUSSION

The vacuolar H^+-ATPase is the most prevalent proton-transporting enzyme of intracellular membranes in plant cells [23, 24]. The H^+-ATPase was identified on the membranes of the endoplasmic reticulum, Golgi apparatus, and endovesicular network; however, it was most commonly found in the tonoplast. Because the pH in these compartments is more acidic, this proton pump is essential for their functional activity. The most important plant cell processes involving the tonoplast V-H^+-ATPase are the generation of the electrochemical potential of hydrogen ions on the vacuolar membrane and the maintenance of cytoplasmic pH homeostasis [17, 24].

It is well known that the effect of endogenous and exogenous factors on plant organisms begins with a decrease in pH. This reaction is so common in various plant species, tissues, and specialized cells, that changes in cytoplasmic H^+ levels can be considered both a signal and a secondary messenger in the transduction of various signaling cascades [25]. This phenomenon has been repeatedly demonstrated in oxygen deficiency [26, 27]. The cytosol acidifies under hypoxia and anoxia for several reasons. The main reason is the low concentration of ATP, which reduces the activity of proton pumps in the plasma membrane and tonoplast [27]. The ATP level in the cell decreases within 1–2 min after switching to anaerobic metabolism [28]. The hydrolysis of ATP and other nucleoside triphosphates (NTPs) is another key source of H^+ [27, 28], as phosphate, pyrophosphate, and nucleoside monophosphate are more acidifying than NTPs. Potential sources of protons include leakage from the vacuole, as well as the accumulation of intermediates and anaerobic metabolism products, primarily lactate [27, 29]. In the tissues of resistant plants, acidosis develops more slowly and is less intense [30, 31]. This may be due to a stronger stimulation of alcoholic fermentation instead of lactic fermentation [29], as well as the presence of alternative metabolic pathways that allow for the reoxidation of NAD(P)H without the accumulation of toxic anaerobic metabolites [1, 29]. As a result, glycolysis transitioning into anaplerotic pathways ensures the production of ATP, which is used to maintain the activity of proton pumps in the plasma membrane and tonoplast. A biochemical pH-stat, comprised of a carboxylating/decarboxylating enzyme shuttle, is also involved in pH regulation [30]. Rice coleoptile cells exhibited acidification of the cytoplasm during anoxia as well [32]. This juvenile organ, capable of germinating when rice is flooded, sharply increases in length to ensure oxygen supply to the submerged tissues of the seedling. This promotes the survival of rice plants, which continue to grow when normal oxygen conditions are restored [33]. During flooding, the growth of the coleoptile in the water is more prolonged than in the air, resulting in greater elongation [34], which is consistent with our findings (Fig. 1). The size of the coleoptile can indicate greater viability of rice plants and is ensured at the genetic level [5, 35].

Our study compared two Russian rice varieties, fast-growing Kuban 3 and slow-growing Amethyst, which differed in the baseline growth intensity of coleoptiles under normal oxygen conditions. The maximum length for both varieties was reported on day 5, and growth ceased thereafter (Fig. 1). The subsequent results indicate that even with hypoxia-induced changes in the growth pattern (growth lasting up to 7 days), the baseline differences in elongation intensity between the varieties persisted. The question is whether all the mechanisms that ensure growth in such drastically differing oxygen conditions will be preserved. Elongation is accompanied by intensive vacuolization. Therefore, the role of the vacuolar

system's marker proton-transporting enzyme is likely to change over time.

Tonoplast V-H$^+$-ATPase is evolutionary similar to F-ATPases in terms of its subunits and the rotational catalysis principle [36]. It consists of 13 subunits arranged into two domains: V_0 and V_1 [24, 37]. There is evidence of the functional significance of various subunits and their potential regulatory mechanisms [16, 24]. However, almost nothing is known regarding the potential role of V-H$^+$-ATPase subunits in elongation under normal oxygen conditions or flooding conditions.

Nevertheless, tissue-specific accumulation during the embryogenesis of Arabidopsis has been reported for the isoforms of the VHA-E subunit, which plays a critical role in the association of the V_1 and V_0 domains of the vacuolar ATPase [38]. The main isoform VHA-E1 is thought to be a housekeeping protein that supports growth. In contrast, the VHA-E2 isoform was identified exclusively in pollen and may be involved in the regulation of pollen tube growth [16, 39]. A number of fragmentary data suggests that the VHA-B subunit, known as nucleotide-binding but having lost its catalytic function, has a significant regulatory role due to its ability to bind to the cytoskeleton and the glycolysis enzyme aldolase [40, 41]. At the same time, the expression of wheat genes encoding this subunit in *Arabidopsis* caused root elongation under salinity stress conditions [42]. Based on the data provided above, these specific subunits were selected for further analysis.

The use of specific antibodies allowed assessing changes in the content of both subunits in the microsomal fraction obtained from rice coleoptile cells (Fig. 2). During aerobic development, the pool of subunit B changed non-linearly and peaked on day 5, when elongation was at its highest. The Kuban 3 variety had significantly more pronounced changes than the Amethyst variety. Interestingly, the same pattern was observed for subunit E. On day 5 of development, its accumulation was almost 18 times higher in the Kuban 3 variety.

Oxygen deficiency during flooding significantly altered the identified pattern. Overall, regardless of rice variety, the content of subunit B remained practically unchanged. Regarding subunit E, there is a slight increase in its abundance compared to the initial stage of development (day 3). There were no further changes in the coleoptile of the fast-growing Kuban 3 variety or the slow-growing Amethyst variety. The results indicate that the involvement of vacuolar H$^+$-ATPase in coleoptile cell growth at different levels of oxygen supply varied. Energy deprivation caused a lack of expected accumulation of subunits B and E not only on day 5, but also on day 7 of development under flooding conditions, when growth was still active.

Thus, the analysis of changes in the expression of genes encoding these subunits is especially important. Notably, VHA-B and VHA-E are encoded by a small family of two genes (*OsVHA-B1, OsVHA-B2* and *OsVHA-E1, OsVHA-E2*). Changes in the accumulation of transcription products of these genes differed significantly from changes in the content of the encoded proteins (Fig. 3). Under normal oxygen conditions, the level of expression remained constant in the vast majority of cases, regardless of variety or coleoptile development stage. One exception is the change in expression of *OsVHA-E2*, peaking on day 5 of development, and *OsVHA-B2*, peaking on day 7 of development. Both exceptions apply to Amethyst seedlings. However, during underwater germination, the majority of genes showed increased expression on day 5 and/or 7, indicating that they may play a role in stress-induced growth. In this scenario, the exceptions were the genes *OsVHA-B2* and *OsVHA-E2*, but only for Kuban 3 seedlings. Several factors can account for these differences. For example, for VHA-E isoforms, a disparity between the accumulation of the RNA product and the concentration of the encoded protein has been reported [43]. Furthermore, there is a possibility of uncoordinated regulation of V-ATPase subunits A and B at the transcriptional level during salinity [44]. Time may also be important. Transcription of the E subunit genes was observed only 72 h after the onset of salinity [45]. Nevertheless, the findings indicate differences in the regulation of expression of genes encoding subunits B and E during the development of two rice varieties with different native growth abilities, under normoxic and hypoxic conditions.

CONCLUSION

In conclusion, the results obtained for rice coleoptiles indicate differences at the expression and proteomic levels for two vacuolar H$^+$-ATPase subunits (B and E) during rice coleoptile cell elongation under normal oxygen conditions and flooding conditions. However, the pattern of growth changes, the prevalence of these subunits in endomembranes, and the intensity of expression of the genes encoding them are not completely similar. This may be due to differences in regulation at the transcriptional and post-translational levels.

Further research is warranted into the functional significance of various vacuolar H$^+$-ATPase subunits in elongation, as well as the mechanisms of temporal coordination of proton-transporting enzyme regulation at the transcriptional and proteomic levels.

ADDITIONAL INFO

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