

H⁺-ATPase functional activity in plant cell plasma membrane

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The aim of the present work was to characterize ATPase activity of wheat coleoptiles cell plasmalemma and check up its coupling with ATP-dependent H⁺ transport under the influence of indole-3-acetic acid and environmental stresses (salt and cold).

ATPase activity was controlled in plasmalemma isolated from four-day-old spring wheat (*Triticum aestivum* L. 'Nandu') coleoptiles by the method of differential ultracentrifugation and purification on sucrose density gradient. Plasmalemma marker enzyme K⁺Mg²⁺-ATPase activity and its suppression by sodium orthovanadate, diethylstilbestrol, dicyclohexylcarbodiimide and inhibitors of possibly contaminating ATPases – oligomycin and nitrate, also proton pumping activities lead to the conclusion that isolated plasmalemma fraction contains Mg²⁺-dependent, K⁺ activated vanadate-sensitive H⁺-ATPase (EC 3.6.1.35).

Artificially created transmembrane electrochemical potential on plasmalemma vesicles activated indole-3-acetic acid influence on plasmalemma ATP-dependent H⁺ transport and response reactions in nuclei. Cold and salt stresses induced changes in coupling of these processes. The data lead to the supposition concerning plasmalemma H⁺-ATPase participation in stresses signals transduction and cell response processes.

Key words: cold and salt stresses, H⁺-ATPase, IAA, plasmalemma.

Introduction. Plant plasma membrane (plasmalemma) proton pump (H⁺-ATPase) is a single polypeptide with molecular mass of about 100 kDa (Arango et al., 2003). It plays a central role in transport processes across the plasmalemma and controls essential functions of plant organism such as nutrient uptake, intracellular pH regulation, cell elongation and leaf movements (Osses, Godoy, 2006). Regulation of the activity of H⁺-ATPase has been proposed to mediate broad range of physiological responses related with growth and development of plants. According to “acid growth” hypothesis indole-3-acetic acid (IAA) enhances H⁺ pumping which lowers cell wall pH, activates pH-sensitive enzymes and proteins within the wall, and initiates all-wall loosening and extension growth. These processes, induced by IAA or fusicoccin, can be blocked instantly and specifically by the removal of K⁺ ions or the addition of K⁺ channel blockers. Vice versa, H⁺ pumping and growth are immediately “switched on” by the addition of K⁺ ions (Hager, 2003). Such data lead to the supposition that these processes could take part in the final realization phases of IAA-dependent plant cell growth by elongation.

Besides the above-mentioned facts, plasmalemma H⁺-ATPase can participate

in early cellular signalling events, as it is an integral plasma membrane protein and for this reason can be significant for perception and transduction of hormonal and environmental signals to cell nuclei.

Broad spectrum of functional activities of H⁺-ATPases in plant cell is associated with large number of enzyme activity regulating factors. Basic function of the enzyme, that of coupling ATP hydrolysis and H⁺-pumping need for fine complex of regulation (Arango et al., 2003) and could be changed as response to different endogen and environmental factors. Among them – phytohormones and changes aroused by signals of environmental stress. The maximal electrochemical potential gradient for H⁺ ($\Delta\mu\text{H}^+$) that can be formed by the H⁺-ATPase is a function of free energy of hydrolysis of ATP and stoichiometry of H⁺ transported per ATP hydrolyzed (Bennet, Spanswick, 1984).

Investigations of functioning of isolated from membrane H⁺-ATPases are possible only after enzyme reactivation and on the models of artificial membranes. More natural and convenient is the model systems created on isolated plasma membrane fragments – sealed vesicles, where ATPases are in their naturally surrounding membrane components. The last model was used in our work.

The aim of the present work was to characterize ATPase activity of wheat coleoptiles cell plasmalemma and check up its coupling with ATP-dependent H⁺ transport under the influence of indole-3-acetic acid and environmental stresses (salt and cold). ATPase activity was controlled in plasmalemma of spring wheat coleoptiles cells intensively growing by elongation – classical test object in phytohormone IAA investigations.

Object, methods and conditions. The tested object of the work – ethyolated decapitated 4-day spring wheat (*Triticum aestivum* L. cv. 'Nandu') coleoptiles. The fraction enriched by sealed plasmalemma vesicles was obtained by the method of differential centrifugation modified for wheat coleoptiles cells. For the identification of H⁺-ATPase in plasmalemma membrane fraction inhibitors of different origin ATPases and their phosphorylated intermediates were used: diethylstilbestrol (50 μM), dicyclohexylcarbodiimide (50 μM), sodium orthovanadate (50 μM), oligomycin (5 mg ml⁻¹). H⁺-ATPase activity in plasmalemma samples was assessed according to accumulation of inorganic phosphate (Pi) (Maksimov et al., 2002). For the evaluation of orientation of plasmalemma vesicles, the total ATPase activity in isolated plasmalemma vesicles was obtained by exposing them to alamethicine (50 $\mu\text{g}\cdot\text{ml}^{-1}$). Plasmalemma vesicle permeability to monovalent cations was determined using potential-sensitive positively charged dye dis-C₃-(5) ($\lambda_{\text{excit.}}$ = 570 nm; $\lambda_{\text{fluor.}}$ = 670 nm; $3.3 \times 10^{-7}\text{M}$). Fluorescence was measured by spectrofluorimeter. Sodium diffusion potential was induced by a specific ionophore valinomycin (8.3 nM) and this served as a starting point for electrochemical potential ($\Delta\mu\text{H}^+$) calculations.

The physiological activity of IAA-protein complexes formed in the plasmalemma (pH 7.2) was evaluated from RNA-polymerase II (RNP-II) activity in a system of nuclei isolated from wheat coleoptiles cells (Кулаева et al., 1979). Triphosphates CTP, GTP, UTP and 8-¹⁴C-ATP (ammonium salt, 2.11 GBq mmole⁻¹, Hartmann Analytic) at final concentration of 0.1 mM were added to the system. The label content was determined with the aid of an LS 1801 scintillation counter (Beckman, USA).

The figures represent mean arithmetical values of 3–5 tests (with no less than 2–3 replications each) and their standard deviations.

Results. 1. Activity of wheat coleoptiles cell plasmalemma H^+ -ATPase. Membrane fraction isolated from wheat coleoptiles cells located in sucrose interphase with $d\ 1.11\text{--}1.15\ \text{g cm}^{-3}$ contained K^+ -activated Mg^{2+} - dependent ATPase. As it is shown in Fig. 1 this ATPase is characterized by exact optimum in acid part of pH 5.5 and high substrate specificity to ATP.

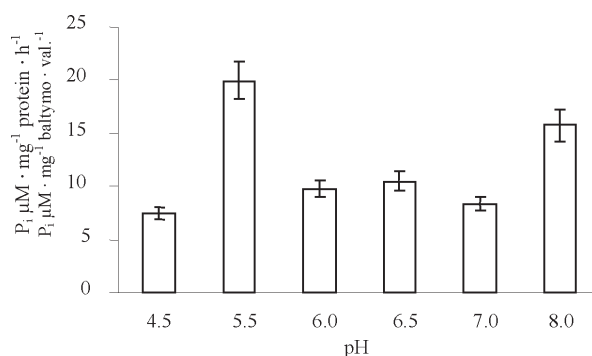


Fig.1. Optimal pH for K^+Mg^{2+} ATPase activity of wheat coleoptiles cell plasmalemma vesicles enriched fraction

1 pav. Kviečių koleoptilių ląstelių plazmolema praturtintos frakcijos ląstelių K^+Mg^{2+} ATPazės aktyvumo optimalus pH

K_m for ATP was $0.3 \pm 0.02\ \text{mM}$, for ADP – $0.9 \pm 0.07\ \text{mM}$. The range of substrates exhibiting the lowering dephosphorylating activity was the following: $\text{ATP} \geq \text{ADP} = \text{paranytrophenylphosphate} \geq \text{AMP} > \text{glycerophosphate}$.

K^+Mg^{2+} -ATPase activity comprised in wheat coleoptile cell plasmalemma fraction was inhibited by sodium orthovanadate, diethylstilbestrol and dicyclohexilcarbodiimide up to 50–73 % (Fig. 2). Nitrate slightly inhibited this ATPase (up to 15 %), oligomycin even at pH 8.0 did not influence it.

These data show that the plasmalemma fractions are slightly contaminated with tonoplast vesicles but without mitochondria contaminations. The investigated K^+Mg^{2+} -ATPase is vanadate sensitive and has phosphorylated intermediates. On the model of sealed vesicles isolated from wheat coleoptiles cell plasmalemma we were able to show the transport function of K^+Mg^{2+} -ATPase and check up the electrochemical potential created due to proton extrusion from the cell imitating processes coupled with K^+ transport to the cell. These data will be showed below. The treatment with alamethycine revealed that the isolated plasmalemma fraction consists from 50 % right side and 50 % inverted vesicles. All above mentioned characteristics of ATPase in plants glycophytes, and wheat among them, are attributed to proton pumping plasmalemma H^+ -ATPase using the energy of ATP hydrolysis (Максимов, 1989).

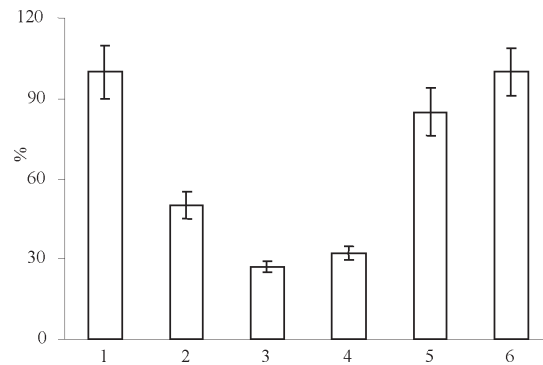


Fig 2. Inhibitor analysis of plasmalemma K^+Mg^{2+} -ATPase activity.

1 – Control, 2 – diethylstilbestrol (50 μ M),

3 – dicyclohexylcarbodiimide (50 μ M), 4 – Na_3VO_4 (50 μ M),

5 – NO_3^- (50 mM), 6 – oligomycin (5 mg ml⁻¹, pH 8.0).

1–5 – pH of incubation medium 5.5

2 pav. Plazmolemos K^+Mg^{2+} -ATPazės inhibitorinė analizė. 1 – Kontrolė,

2 – dietilstilbestrolis (50 μ M), 3 – dicikloheksilkarbodiimidis (50 μ M),

4 – Na_3VO_4 (50 μ M), 5 – NO_3^- (50 mM), 6 – oligomicinas (5 mg ml⁻¹, pH 8,0).

1–5 – Inkubacinės terpės pH 5,5

2. IAA and H^+ -ATPase functional activity. IAA treatment *in vivo* activated wheat coleoptiles cell growth by elongation. IAA used *in vitro* slightly raised transmembrane ion transport in plasmalemma vesicles. The latter processes were expressed in more considerable extent (up to 22 %) when treatment with IAA underwent in experimental conditions favouring the creation of IAA-protein plasmalemma complexes (Fig. 3). IAA-dependent processes undergoing in the cell nuclei do not take part in the models with isolated plasmalemma vesicles, which were used for studies of electrochemical cations gradient formation. Consequently here we have only effects of IAA on plasmalemma level without any IAA-dependent protein synthesis activation. Then the question arises whether IAA created ion transport changes on plasmalemma level participate in nuclei IAA-dependent responses. The plasmalemma H^+ -ATPase extrudes H^+ from cell to generate a proton motive force with membrane potential of -120 to -160 mV (negative inside) and pH gradient of 1.5 to 2 units (acid outside) (Sze et al., 1999). We found the abilities to create artificially transmembrane potential on the plasmalemma vesicles: by the method elaborated by Maksimov (1989) plasmalemma vesicles loaded by K^+ ions were transferred into a Na^+ -medium and K^+ permeability on membrane induced by valinomycin.

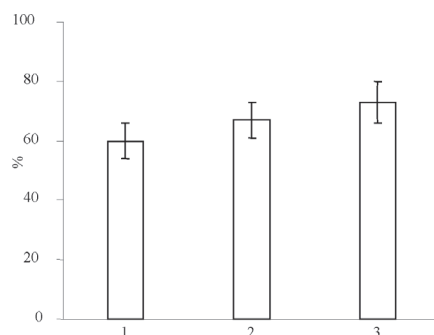


Fig. 3. Influence of IAA ($5 \cdot 10^{-8}$ M) on the active proton transport through membrane of plasmalemma vesicles according to fluorescence of dis - C₃ - (5).

1 – Control, 2 – IAA, $5 \cdot 10^{-8}$ M,

3 – IAA, $5 \cdot 10^{-8}$ M + the protein fraction of plasmalemma (~ 20 kDa). 100 % – K⁺ – diffusion potential.

3 pav. IAR ($5 \cdot 10^{-8}$ M) poveikis aktyviam protonų transportui per plazmolemą pagal dis - C₃ - (5) fluorescenciją. 1 – Kontrolė, 2 – IAR, $5 \cdot 10^{-8}$ M,

3 – IAR, $5 \cdot 10^{-8}$ M + plazmolemos baltymų frakcija (~ 20 kDa).
100 % – K⁺ – difuzinis potencialas.

The transmembrane potential calculated by titration according to the Nernst equation in such conditions reaches about 100 mV (Maksimov, 1989). The generated K⁺-diffusion potential was checked by potential-sensitive dye dis-C₃-(5). IAA-dependent changes in the nuclei were studied according to the changes of RNR-polymerase II activity in the system of isolated nuclei. The obtained data have shown that the addition to the system of the plasmalemma fraction treated with IAR to the enzyme activity provoking medium arouse more pronounced activity of RNA-polymerase II under conditions of artificially created electrochemical gradient (Fig. 4). These data allow the suggestion that electrochemical gradient on membrane which naturally creates at a great deal by functioning of H⁺-pump could influence processes of IAA perception and realization.

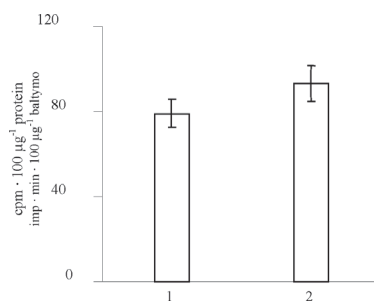


Fig. 4. Activity of RNA-polymerase II in the system of isolated nuclei with addition of IAA ($5 \cdot 10^{-6}\text{M}$) treated plasmalemma vesicles (1 and 2).

1 – Electrochemical potential not created on plasmalemma vesicles,

2 – Under conditions of created electrochemical gradient

4 pav. RNR-polimerazės II aktyvumas izoliuotų branduolių RNR sistezės sistemoje, pridėjus IAR ($5 \cdot 10^{-6}\text{M}$) paveiktų plazmolemos vezikulių (1 ir 2).

1 – be elektrocheminio potencialo plazmolemos vezikulėse,

2 – sukurto elektrocheminio potencialo sąlygomis

3. Hydrolytic and transport functions of plasmalemma H^+ -ATPase under conditions of environmental stresses. Changes in plasmalemma H^+ -ATPase dephosphorylating activity and H^+ transport are main markers of functional state of this membrane. Could the environmental stresses, for example, cold and salt stresses, arise the changes in plant cell plasmalemma related with the maintaining of plant cell stability directed to its survival under unfavourable conditions? For answering this question we restricted this very broad field of investigations by determination of H^+ -ATPase functioning properties under two fixed conditions – one hour long treatment by $-8\text{ }^\circ\text{C}$ or 250 mM NaCl .

The changes in plasmalemma H^+ -ATPase activity under the above-mentioned environmental stresses are shown in Fig. 5. Sub lethal treatment *in vivo* by cold ($-8\text{ }^\circ\text{C}$) exhibited only tendency for altering of dephosphorylating activity in wheat coleoptiles cell plasmalemma, but short treatment of coleoptiles by 250 mM NaCl lowered it up to 17 %. At the same time we were not able to elucidate pronounced changes in plasmalemma H^+ transport processes. $\Delta\mu\text{H}^+$ of plasmalemma in cold treated coleoptiles was slightly lowered, but more interesting situation was exhibited in salt treated variants – there was no lowering of $\Delta\mu\text{H}^+$ in spite of the fact that ATPase dephosphorylating activity, as it was shown in Fig. 5, was inhibited up to 17 % (Fig. 6).

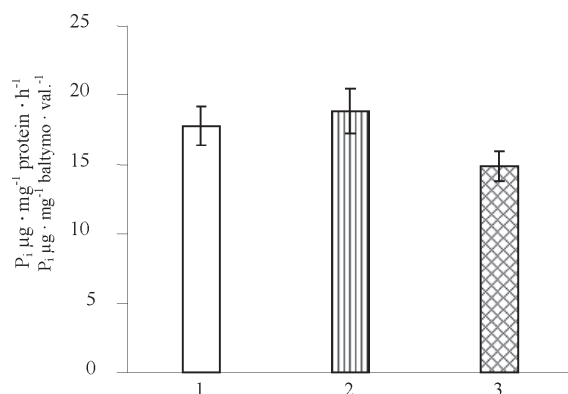


Fig. 5. Activity of wheat coleoptile cell plasmalemma H⁺-ATPase. 1 – control, 2 – -8 °C, 1 h, 3 – 250 mM NaCl, 1 h. All treatments *in vivo*.
5 pav. Kviečių koleoptilių ląstelių plazmolemos H⁺-ATPazės aktyvumas. 1 – kontrolė, 2 – -8 °C, 1 val., 3 – 250 mM NaCl, 1 val. Visi poveikiai *in vivo*.

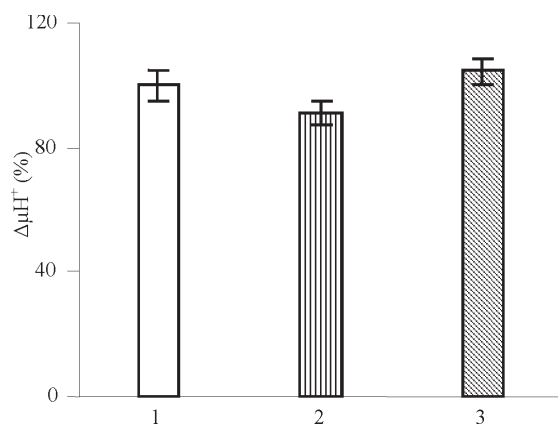


Fig. 6. ATP-dependent electrochemical potential ΔμH⁺ in wheat coleoptiles plasmalemma vesicles. 1 – control, 2 – -8 °C, 1 h, 3 – 250 mM NaCl, 1 h. All treatments *in vivo*.
6 pav. Nuo ATP priklausomas elektrocheminis potencialas ΔμH⁺ kviečių koleoptilių plazmolemos vezikulėse. 1 – kontrolė, 2 – -8 °C, 1 val., 3 – 250 mM NaCl, 1 val. Visi poveikiai *in vivo*.

So, the above data show that plasmalemma properties, which are directly related with coleoptiles cell homeostasis, even at sub lethal stress conditions could be maintained at the optimal level for cell living processes.

Discussion. The studies described above were planned in order to show how wheat coleoptile cell plasmalemma H⁺-ATPase activity is exhibited in two main fields of possible participation in the processes cell growth and responses to sublethal stresses.

Highly purified plasmalemma fraction was used in the studies. According to inhibitory analysis of K^+ -activated Mg^{2+} -dependent ATPase activity, slight contamination with tonoplast vesicles (slight inhibition by nitrate) and no contamination by ATPases from mitochondria (no inhibition by oligomycin) were determined. Activity, pH optimum, specificity to ATPase as substrate, functioning peculiarities, sensitivity to orthovanadate and dicyclohexylcarbodiimide lead us to the conclusion that we have elucidated the plasmalemma H^+ -ATPase – H^+ pump, which according to literature data is vanadate sensitive K^+ -activated Mg^{2+} -dependent ATPase (EC 3.6.1.35) located in plant plasmalemma (Osses, Godoy, 2006).

One of the main functions of plasmalemma H^+ -ATPase is H^+ extrusion from cell cytosol to cell wall area and loosening of cell wall microfibrils leading to cell growth by elongation (Hager, 2003). These processes can be controlled by IAA and fusicoccine and possibly reveal the last phases of hormone controlled growth realization. More complicated are the situations with early events of growth and development controlling processes, which could be influenced by changing plasmalemma H^+ -ATPase activity. It is well known that in plasmalemma of functioning cells 100–150 mV transmembrane potential is generated and maintained. Many if not all processes taking place in plasmalemma are dependent on this transmembrane potential. The last is maintained by the work of H^+ pump, functioning of ion channels (for example – K^+ and Ca^{2+} channels), cell growth-regulating processes. Plasmalemma H^+ -ATPase activity is closely related with seasonal course of plant growth: plasmalemma H^+ -ATPase activity in *Festuca pratensis* Huds showed specific fluctuation when ATPase activity peaks were determined early in spring – just before renewal of growth, and the lowest ATPase activity was in root at the time approaching to flowering and in shoot basal nodes during the transition to growth cessation (Darginavičienė et al., 2007). There are literature data about plasmalemmal regulator protein 14-3-3 interaction with H^+ -ATPase, which leads to strong stimulation of H^+ -ATPase activity. The processes are interrelated with phosphorylation events in plasmalemma (Garufi et al., 2007) during primary signal transduction steps.

Plant H^+ -ATPase can also be regulated independently of 14-3-3 proteins. An auxin-binding protein isolated from rice was found to stimulate H^+ -ATPase activity directly, and auxin binding increased the affinity of the auxin-binding protein for H^+ -ATPase (Arango et al., 2003). There is no definite opinion about direct influence of IAA on H^+ -ATPase activity. According to Erdel (1979) very small IAA concentrations (10^{-10} – 10^{-8} M) induced ATPase activity of microsomal fraction. In the work with wheat it was suggested that plasmalemma H^+ -ATPases is a component of IAA signalling cascade that may direct pattern formation in embryos (Rober-Kleber et al., 2003). Our data show that in wheat coleoptiles (growing by elongation) transmembrane ATP dependent potential is able to activate IAA response reactions in nuclei. These data are also in agreement with possible role of plasmalemma H^+ -ATPase functioning in the first phases of IAA signal perception and transduction processes.

Changes in plasmalemma H^+ -ATPase activity was observed as response to cold stress. So when cucumber under exposure at 8 °C temperature for 1 day reduced plasma membrane H^+ -ATPase activity from 30 to 16 $\mu\text{mol } P_i \text{ mg}^{-1} \text{ protein } 1 \text{ h}^{-1}$ (Lee et al., 2004). The mentioned changes could be attributed to indirect enzyme activity changes to temperature lowering, but there are suppositions that cold-induced reactive

oxygen species may activate a mitogen activated protein-kinase cascade that regulates tolerance to freezing and other abiotic stresses.

Cucumber seedlings treated by 200 mM NaCl one day showed increased activity of plasmalemma and tonoplast H⁺-ATPases which correlated with altered H⁺ transport. The alignment between H⁺-ATPase functional processes – enzyme hydrolytic activity and ATP-dependent H⁺ transport – could undergo changes (Bennet, Spanswick, 1984). Such changes could be observed under the influence of different stress conditions. Moreover our investigations with cold tolerant and non-tolerant *Festuca pratensis* Huds genetic lines revealed that cold tolerance is characteristic for *Festuca* plant in the cell plasmalemma –membranes of cold tolerant lines are able to maintain H⁺ transport level ensuring its homeostasis in spite of cold stress dependent changes in ATPase dephosphorylating activity (Darginavičienė et al., 2007). Partial confirmation achieved in present work is that spring wheat coleoptiles, which usually can be influenced by spring cold stresses, are able to stabilize H⁺ transport levels.

Conclusions. 1. Plasmalemma fraction isolated from spring wheat coleoptiles contains Mg²⁺-dependent, K⁺-activated vanadate sensitive H⁺-ATPase – proton pump.

2. Artificially created transmembrane electrochemical potential activated an IAA influenced ATPase dependent H⁺ transport in plasmalemma and response reactions in nuclei.

3. The supposition is made that cell plasmalemma of more tolerant for environmental stresses plants is able to change the coupling between ATPase hydrolytic and H⁺ transport activities and so maintain the H⁺ transport levels ensuring the cell homeostasis.

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Funkcinis augalų ląstelių plazmolemos H⁺-ATPazės aktyvumas

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Santrauka

Pateikiamo darbo tikslas – charakterizuoti ATPazės hidrolitinį aktyvumą vasarinių kviečių koleoptilių plazmolemoje ir jo santykį su nuo ATP-priklausomu H⁺ transportu indolil-3-acto rūgšties ir išorinių stresų (druskų ir šalčio) poveikyje.

Darbui naudotos keturių parų etioliuotos kviečių (*Triticum aestivum* L., 'Nandu') koleoptilės, iš kurių ląstelių diferencinio ultracentrifugavimo būdu buvo išskiriama plazmolema ir išvaloma sacharozės tankio gradientu. Plazmolemos markerinio fermento K⁺Mg²⁺-ATPazės aktyvumas ir natrio ortovanadato, dietilstilbestolio, dicikloheksilkarbodiimido bei galimų užterpiančių ATPazių inhibitorių (oligomicino ir nitrato) poveikis, o be to, protonų perneptimo per membrana ypatybės leido padaryti išvada, kad išskirtoji plazmolemos frakcija talpina nuo Mg²⁺-priklausomą, K⁺-aktyvuojamą vanadatui jautrią H⁺-ATPazę (EC 3.6.1.35).

Dirbtinai sukurtas plazmolemos vezikulėse elektrocheminis potencialas sustiprino indolil-3-acto rūgšties poveikį plazmolemos ATP-priklausomam H⁺ transportui ir atsako reakcijoms branduolyje. Šalčio ir druskų stresai sukėlė pakitimus santykyje tarp ATPazės hidrolitinio aktyvumo ir ATP-priklausomo H⁺ transporto ir parodė, kad augalų ląstelių plazmolemos H⁺-ATPazė gali dalyvauti stresinių signalų transdukcijos ir atsako į juos procesuose.

Reikšminiai žodžiai: šalčio ir druskų stresai, H⁺-ATPazė, IAR, plazmolema.