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INHIBITORS OF TYROSINE KINASES AND PHOSPHATASES AS A TOOL FOR THE INVESTIGATION OF MICROTUBULE ROLE IN PLANT COLD RESPONCE

Tyrosine phosphorylation plays a vital role in the variety of signal transduction pathways in eukaryotic cells, however its role and relevance in plants are still largely unknown. To investigate the functional role of tubulin tyrosine phosphorylation in plant cells the interplay between the effects of tyrosine kinases (herbimycin A) as well as tyrosine phosphatases (sodium orthovanadate) inhibitors on microtubules sensitivity to cold in A. thaliana root cells were studied. Since it was found that inhibition of tyrosine kinases significantly increased the microtubules sensitivity to cold, while inhibition of tyrosine phophatases enhanced their cold-resistance, we suggest an existence of certain functional interaction between the phosphorylation on tyrosine residues and sensitivity of cortical microtubules to low temperatures.

Introduction. Microtubules (MTs) are essential cytoskeleton elements involved in numerous fundamental processes in plant cells [1]. The diverse functions of MTs are defined by the dynamic properties of MTs as well as by distribution and arrangement of MTs through out the cell [2, 3]. Plants have distinct gene sets coding main structural MTs protein (α- and β-tubulin) even for MTs arrays apparently similar in structure [4]. It is known that a specific expression of tubulin genes does exist under different developmental stages and environmental conditions [5–9]. Tubulin composition varies both spatially and temporally by transcription of different tubulin genes that is further diversified by the mechanisms of post-translational modifications (PTMs) used for labeling of MTs subpopulations [10].

It was found that both α- and β-subunits of plant tubulin are post-translationally modified [11–17]. However, for the most PTMs of plant tubulin the functional importance needs to be elucidated. Recently using biochemical approaches we have shown that both plant tubulin subunits (α- and β-) are phosphorylated on tyrosine residues [16]. Immunofluorescence microscopy revealed that tyrosines in polymerized MTs are indeed phosphorylated, and this phosphorylation occurs on both cortical and mitotic MTs in *Arabidopsis* cells [18, 19]. Our experiments with tyrosine kinase and phosphatase inhibitors identified also tyrosine phosphorylation as an important factor in regulation of MTs organization in *A. thaliana* primary root cells [20]. However, the functional significance of MTs phosphorylation on tyrosine residues with impact on MTs organization and stability, including responses to various external stimuli, remains an open issue to date.

For instance, it is know that temperature is an important factor that affects MTs organization and dynamics in plant cells [21]. Several studies have shown that plant MTs disassemble in response to low temperature [22–24]. Depolymerization of MTs has been linked to cold induced gene expression and activation of the low temperature signalling pathway [25]. Differential expression of plant tubulin genes during cold treatment have been

observed, but relatively little information is available on functional role of tubulin PTMs as one of the potential important component of signal transduction mechanism in higher plants. However, it has been demonstrated that MTs behavior can be influenced by protein phosphorylation process [26–29] as a set of serine/threonine protein kinases inhibitors can modify MTs stability under low temperature [22, 24].

Thus, the aim of present research was to investigate MTs organization in response to cold treatment, and estimate whether the interplay between the phosphorylation/dephosphorylation processes and MTs sensitivity to cold exposure does exist in plant cells. Here we report that inhibition of tyrosine kinases significantly increases plant MTs sensitivity to cold. As opposed to that cell treatment with tyrosine phosphatase inhibitor resulted in resistance enhancement of cortical MTs to cold as compared to control. Obtained results allow us to suggest an existence of certain functional interaction between the tubulin phosphorylation on tyrosine residues and sensitivity of cortical MTs to cold treatment in *A. thaliana* primary root cells.

Materials and methods. *Plant material.* Seedlings of *Arabidopsis thaliana* (Landsberg erecta (Ler) ecotype) expressing *gfp-map4* [30] were grown under aseptic conditions as described earlier [20]. Four-day-old seedlings were used for the experiments. Cold treatment were administered either as seedlings exposition at +0,5 °C for time period during 1–5 h.

Chemical treatment. Stock solutions of herbimycin A (inhibitor of non-receptor tyrosine kinases) and sodium orthovanadate (inhibitor of tyrosine phosphatases) were prepared as described earlier [20]. For the study of the combined effects of tyrosine kinases or phosphatases inhibitors and low temperature *A. thaliana* seedling were pretreated firstly with herbimycin A (50 μM) or sodium orthovanadate (250 μM) at room temperature (+23 °C) during 1 or 2 h and than transferred to cold chamber (+0,5 °C) for 1–5 h.

Confocal microscopy. Confocal imaging was performed using a LSM 510 META confocal laser scanning microscope («Carl Zeiss», Germany) with a Zeiss 63× Plan-Apochromat NA 1.4 oil-immersion objective lens. Green fluorescent protein (GFP) excited with the 488 nm wavelength of an argon laser (at 15 % power output) with emission collected by the BP 510–570 nm filter. Immediately following treatments the seedlings were transferred on glass coverslips and cells of meristematic, transition, elongation as and differentiation zones of *A. thaliana* primary root were scanned to observe MTs organization. The effects of inhibitors and cold shock treatments on MTs organization in different cell types were examined from 15 different plants. Each set of treatments and observations were preformed in at least three experiments.

Results. *MTs response to cold.* At first the influence of low temperature on cortical MTs organization in cells of different growth root zones of *A. thaliana* seedlings were investigated. Typical organization of cortical MTs in cells of *A. thaliana* (GFP–MAP4) primary root were described in details earlier [31] and it is shown in Fig. 1, *a–c*. It was found that cold exposure of *A. thaliana* seedlings during 1 and 2 h caused no obvious changes in cortical MTs orientation and organization in cells of meristematic, elongation as well as differentiation zones of primary roots (Fig. 2, *a*, *c*) as compared to control (Fig. 1, *a–c*). Whereas in some cells of transition zone MTs less amount of MTs were visualised (Fig. 2, *b*) as compared to control (Fig. 1, *b*).

Seedlings subjected to low temperature during >3 h revealed dramatic changes in MTs organization. For instance, cold exposure during 4 h caused significant fragmentation of cortical MTs in the majority of cells of meristematic zone (Fig. 2, *d*). At the same time MTs in epidermal and cortex cells of transition and elongation as well as differentiation zones completely lost their organized structure (Fig. 2, *e*, *f*). Moreover, after 5 h seedlings exposure under low temperature cortical MTs in all types of *A. thaliana* root cells were depolymerised.

Tyrosine kinases and tyrosine phosphatases inhibitors impact on cortical MTs sensitivity to cold. It was established that the treatment of *A. thaliana* seedlings with 50 μM herbimycin A during 1 and

2 h caused no changes on MTs organization in all types of primary root cells (Fig. 3, *a–c*). However our previous data suggest that only 4 h treatment with herbimycin A at the same concentration resulted in MTs depolymerization in cells of differentiation zone, whereas MTs in epidermal cells of the meristematic, transition as well as elongation zones were less sensitive to herbimycin A treatment [20].

It was found that *A. thaliana* seedlings pretreatment with herbimycin A for 1 h and their further exposure to cold during 1 or 2 h provoked dramatic changes in MTs organization. MTs disorientation and depolymerization in some epidermal cells of meristematic zone (Fig. 3, *d*) as well as in the most epidermal cells of transition and elongation zones (Fig. 3, *e*) were found while in the cells of differentiation zone MTs were partially or totally depolymerized (Fig. 3, *f*).

However, in cells of seedlings exposed to cold only during the same time (1 or 2 h) were no visible changes of MTs organization (Table). It should be noted that 2 h seedlings pretreatment with herbimycin A and further cold exposure during 1 h led to complete cortical MTs depolymerization in epidermal cells of all primary root growth zones (Table). Therefore, it is likely that inhibition of tyrosine kinases significantly increases the cortical MTs sensitivity to cold.

On the other hand, *A. thaliana* seedlings treatment with 250 μM sodium orthovanadate only (inhibitor of tyrosine phosphatases) during

Effect of herbimycin A, sodium orthovanadate and cold on MTs organization in cells of *A. thaliana* roots

Inhibitor	Treatment, h		Root growth zone									
			Root tip			Transition		Elongation		Differentiation		
	inhibitor, 23 °C	cold, 0.5 °C	root cap	epidermal	meristematic	epidermal	cortex	epidermal	cortex	epidermal	cortex	root hairs
–	–	1	+	+	+	+	+	+	+	+	+	+
	–	2	+	+	+	+/–	+	+	+	+	+	+
	–	3	+/–	+/–	+/–	–	+/–	+/–	+/–	+/–	+/–	+/–
	–	4	+/–	+/–	–	–	–	–	–	+/–	–	–
	–	5	–	–	–	–	–	–	–	–	–	–
Herbimycin A	1	–	+	+	+	+	+	+	+	+	+	+
	1	1	+/–	+/–	+/–	+/–	+/–	+/–	+/–	+/–	–	–
	1	2	–	+/–	–	–	–	–	–	–	–	–
	2	–	+	+	+	+	+	+	+	+	+	+
	2	1	–	–	–	–	–	–	–	–	–	–
Sodium orthovanadate	1	–	+	+	+	+	+	+	+	+	+	+
	1	1	+	+	+	+	+	+	+	+	+	+
	1	2	+	+	+	+	+	+	+	+	+	+
	1	5	+	+	+	+	+	+	+	+	+	+
	2	–	+	+	+	+	+	+	+	+	+	+
	2	1	+	+	+	+	+	+	+	+	+	+
	2	2	+/–	+	+	+	+	+	+	+	+	+
	2	5	+/–	+	+	+/–	+	+/–	+	+	+	+

Indications. «+» – normal MTs organization; «+/–» – partial MTs depolymerization, «–» – MTs could not be visualized.

1 and 2 h did not affect MTs organization in primary root cells (Fig. 4, *a–c*). Pretreatment of *A. thaliana* seedlings with sodium orthovanadate (1 or 2 h) and their further exposition to cold during 1 and 2 h have not caused alterations in MTs organization as well (Table). Nevertheless, after further cold exposure of *A. thaliana* seedlings during 3, 4 and 5 h in the majority of cells of meristematic, transition, elongation and differentiation zones MTs were visualized (Fig. 4, *d–f*). Though, MTs in cells of the same *A. thaliana* primary growth zones exposed only to cold during the 3, 4, 5 h were shortened or totally depolymerized as it was described above. Thereby these results indicate that *A. thaliana* seedlings pretreatment with inhibitor of tyrosine phosphatases increases stability of cortical MTs to low temperature treatment.

Effects of tyrosine kinases and tyrosine phosphatases inhibitor treatments as well as low temperature on MTs organization in cells of *A. thaliana* primary root are summarized in the Table.

Discussion. Our data clearly demonstrate that MTs in cells of *A. thaliana* primary root zones have different sensitivity to low temperature exposure. The most sensitive to cold were MTs in cells of transition zone, whereas the most stable were MTs in cells of meristematic zone. These results are in accordance with other data showing that MTs in cells of various maize root tissues at different distances from apex exhibited diverse sensitivity to cold [32]. It was established that MTs in cells of post-mitotic and rapidly elongating zones of the maize root apex (transition zone) were more sensitive to low temperature than MTs in cells of the meristem, as well as in cells of the proximal portion of the elongation zone, where cell elongation was slow [32]. Recently it was found that transition zone integrates diverse inputs from endogenous and exogenous stimuli and translates them into signalling and motoric outputs as adaptive differential growth responses [23]. Generally MTs in cells of transition zone characterized with highly dynamic parameters of MT polymerization are highly sensitive to the range of different factors like gravity [33], UV-B irradiation [32], phytohormones [32], serine/threonine protein kinase and phosphatase inhibitors [20, 28, 29]. According to our data as well as results of other research groups it could be assumed that MTs, especially in transition zone, could play an integral role in perception of different signals that enable plants adapt to environmental changes, including cold.

Reversible protein phosphorylation is one of the most common means of signal transduction pathways in plants, including cold stress-response (freezing) and cold-acclimation (chilling) pathways [35, 36]. Recently we have found that phosphorylation of MTs proteins, primarily tubulin, on serine/threonine residues can be involved in cell cycle progression [27], primary root elongation processes as well as induction, formation and growth of root hairs in *A. thaliana* seedlings [29]. Furthermore protein phosphorylation on serine/threonine residues could participate in regulation of MTs cold stability in plant cells [22, 24].

Until recently the importance of phosphorylation on tyrosine residues has been largely neglected because typical tyrosine kinases were not found in plants. However, specific tyrosine phosphatases do exist in higher plants and play a key role in some physiological processes in plant cells [37–39]. It was also established that tyrosine phosphorylation participates in different cellular processes in plants like disease-resistance signalling [40, 41], MAP kinase cascades [42] as well as other processes in plant cell signaling [43–47].

Bioinformatics approaches allowed us to demonstrate the similarity of some *Arabidopsis* proteins with the unknown functions to the profiles of animal tyrosine specific protein kinases [48]. Moreover, in green alga the tyrosine kinases (Syk-like) with the putative key role in growth and development were identified [49].

Recently we have shown also that plant tubulin is phosphorylated on tyrosine residues [16]. Immunofluorescence microscopy revealed that tyrosine phosphorylation of β-tubulin [18] as well as α-tubulin [19] could be one of the targets

for tyrosine kinases. Homological modeling of tubulins in *A. thaliana* estimated that tyrosines in C-terminal region of α- and β-tubulin subunits are exposed on the MT outer side that makes them the candidates for the potential targets of tyrosine kinases [18]. Analysis employing tyrosine kinase and phosphatase inhibitors allowed us to assume that tyrosine phosphorylation/dephosphorylation processes could be involved in the regulation of growth and development of *A. thaliana* roots as well as in the regulation of overall MTs organization in different cell types [20].

In present study we primarily established the functional interaction between the inhibition of tyrosine kinases/phosphatases activity in plant cells and cortical MTs sensitivity to cold. It was found that *A. thaliana* seedlings pretreatment with tyrosine kinases inhibitor significantly increase the cortical MTs sensitivity to cold as MTs in cells of differentiation zone of *A. thaliana* almost completely depolymerized after 1 h exposure in cold conditions. Whereas in control seedlings after 1 h exposure at the same low temperature MTs in cell of all growth zones of the *A. thaliana* root preserve initial organization. As opposed to that after root pretreatment with tyrosine phosphatase inhibitor cortical MTs in *A. thaliana* cells became more resistant to cold exposure as compared to control exposed only to cold.

Thus, summarizing our data obtained we can suppose that phosphorylation of plant tubulin on tyrosine residues can be one of the mechanisms of MTs sensitivity/stability to low temperature exposure and could input in to cold acclimatization process.

This assumption relies on our previous data showing that in *Daucus carrota* suspension cells anti-phosphotyrosine antibody PY-20 stained in cold-stable MTs fraction the dominant proteins corresponds to the position of α–β-tubilin dimers [16]. We can speculate also that tyrosine phosphorylation may affect MTs binding to microtubule associated proteins or motor proteins that can input into MTs cold stability/sensitivity. The next challenge would be to identify the protein kinases that participated in MTs protein phosphorylation on tyrosine residues and define the possible functional role of this process in plant cells.

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ИСПОЛЬЗОВАНИЕ ИНГИБИТОРОВ ТИРОЗИНОВЫХ КИНАЗ И ФОСФАТАЗ ДЛЯ ИССЛЕДОВАНИЯ РОЛИ МИКРОТРУБОЧЕК В ОБЕСПЕЧЕНИИ ОТВЕТА РАСТЕНИЙ НА ХОЛОД

Фосфорилирование остатков тирозина является важным компонентом сигнальных каскадов в эукариотических клетках, однако его роль и значимость в растениях остается практически неизвестной. Для того чтобы выяснить функциональную роль тирозинфосфорилирования тубулина в растительной клетке, исследовали взаимосвязь между влиянием ингибитора тирозинкиназ (гербимицина А) и тирозинфосфатаз (ортованадата натрия) и чувствительностью микротрубочек в клетках корня *A. thaliana* к действию холода. Поскольку было выявлено, что ингибирование тирозинкиназ значительно увеличивает чувствительность микротрубочек к холоду, а ингибирование тирозинфосфатаз повышает их холодоустойчивость, мы предполагаем наличие определенной функциональной взаимосвязи между фосфорилированием по остаткам тирозина и чувствительностью кортикальных микротрубочек к действию холода.

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ВИКОРИСТАННЯ ІНГІБІТОРІВ ТИРОЗИНОВИХ КІНАЗ І ФОСФАТАЗ ДЛЯ ДОСЛІДЖЕННЯ РОЛІ МІКРОТРУБОЧОК У ЗАБЕЗПЕЧЕННІ ВІДПОВІДІ РОСЛИН НА ХОЛОД

Фосфорилювання залишків тирозину є важливим компонентом сигнальних каскадів в еукаріотичних клітинах, проте його роль і важливість в рослинах дотепер залишається практично невідомою. Щоб виявити функціональну роль тирозинфосфорилювання тубуліну в рослинній клітині, досліджено взаємозв'язок між впливом інгібіторів тирозинкіназ (гербіміцину А) і тирозинфосфатаз (ортованадату натрію) та чутливістю мікротрубочок в клітинах кореня *A. thaliana* до дії холоду. Оскільки було встановлено, що інгібування тирозинкіназ значно збільшує чутливість мікротрубочок до холоду, тоді як інгібування тирозинфосфатаз підвищує їхню холодостійкість, ми припускаємо існування певного функціонального взаємозв'язку між фосфорилюванням по залишках тирозину та чутливістю кортикальних мікротрубочок до низьких температур.

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