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INDOLE-3-ACETIC ACID-PROTEIN COMPLEXES IN CHLOROPLASTS AND MITOCHONDRIA

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VILNIAUS UNIVERSITETAS
GAMTOS TYRIMŲ CENTRO BOTANIKOS INSTITUTAS

Rima Mockevičiūtė

INDOLIL-3-ACTO RŪGŠTIES-BALTYMŲ KOMPLEKSAI CHLOROPLASTUOSE IR MITOCHONDRIJOSE

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INTRODUCTION

Plant hormone indole-3-acetic acid (IAA or auxin) alone or together with other phytohormones regulates many physiological processes. Control of elongation, division and differentiation are attributed to IAA at the level of the cell (Меркис, 1982; Jones et al., 1998; Teale et al., 2006). Although IAA is the first hormone detected and identified in plants, however, it has not been disclosed yet how chemically simple IAA molecule can determine or influence such wide range of plant growth and development processes (Merkys et al., 2003; Theologis, 2004; Mockaitis, Estelle, 2008). The molecular mechanism of IAA function is known to begin by the interaction with auxin recognizing and specifically binding proteins – ABP (Bonner, 1965; Merkys, 1966), whereas the impact of formed complexes is directed towards the modification of genetic information in nucleus (Merkys et al., 1988; Darginavičienė et al., 1991; Abel, Theologis, 1996; Leyser, 2002). Therefore, ABP are undoubtedly essential components of the molecular mechanism of IAA function, predetermining physiological response (Меркис, 1982; Badescu, Napier, 2006).

For a long time, the investigations on a specific IAA-ABP interaction were focused on plasmalemma of monocotyledonous and dicotyledonous plants (Venis, 1977 a; Ray et al., 1977 a; Hertel, 1979; Merkys et al., 1988; Даргинавичене, 1992; Anisimovienė et al., 2004; Jodinskienė, 2005). Presently, the IAA receptor ABP1 – a protein mediating rapid monocotyledonous plants' cell response to this phytohormone by elongation growth has been finally identified (Venis et al., 1992; Woo et al., 2002; Napier, 2004), however, the pathway of its signal transmission into nucleus has not been disclosed yet (Teale et al., 2006; Christian et al., 2006). A protein performing analogous function in wheat coleoptiles plasmalemma was also detected (Merkys et al., 1988; Даргинавичене, 1992). The same IAA binding site – /-His-Arg-His-Ser-Cys-Glu-/ and function is characteristic of ABP26 that was also identified in dicotyledonous plant kidney bean elongating hypocotyls cells plasmalemma (Anisimovienė, Merkys, 2000). Intracellular auxin receptor TIR1 – a soluble protein in nucleus has recently been identified as mediating auxin-regulated transcription, which determines the development of a plant (Dharmasiri et al., 2005; Kepinski, Leyser, 2005). Other ABPs were also identified in plant cell plasmalemma, cytosol and tonoplast. Some of them were identified as proteins

with enzymatic activities; others were characterized as possible auxin receptors or transporters (Oostrom et al., 1980; Zaina, 1989; Macdonald et al., 1991; Даргинавичене, 1992; Merkys, Darginavičienė, 1997), whereas the functions of a number of other ABP localized in these compartments of cell have not been investigated yet.

Meanwhile, the questions – whether ABP might be localized in other compartments of cell and what kind of role might they play in the realization of IAA physiological function – have not been answered yet or have not been discussed at all (Darginavičienė, Novickienė, 2002; Merkys et al., 2003; Woodvard, Bartel, 2005; Anisimovienė et al., 2006). It is known that IAA is localized/synthesized in plant cell organelles – chloroplasts and mitochondria as well as in their bacterial ancestors (Anisimovienė, 1974; Fregeau, Wightman, 1983; Ohmiya, Hayashi, 1992; Sandber et al., 1990; Sergeeva et al., 2002) however, its physiological role with respect to organelle and the whole cell has not been studied yet. Hence, that induced the search of ABP particularly in those organelles – chloroplasts and mitochondria.

Aim of the research was to establish a possibility of ABP presence and functioning in kidney bean cell organelles – chloroplasts and mitochondria.

Main tasks:

- To ascertain a possibility of IAA recognizing and specifically interacting proteins localization and functioning in kidney bean leaf cell intact chloroplasts and intact mitochondria of hypocotyls cells zone, responding to IAA by elongation growth.
- To reveal the peculiarities of IAA and ABPs interactions in chloroplasts and mitochondria and to characterize the forming complexes.
- To establish ABP localization in the sub-compartment(s) of chloroplasts and mitochondria.
- To characterize specific IAA-ABP complexes forming in the sub-compartments of chloroplasts and mitochondria, as well as ABP (individual proteins) forming them.
- To compare IAA-ABP complexes forming in chloroplasts and mitochondria with IAA-ABP complexes forming in other cell compartments.

Scientific novelty and theoretical significance of the research. For the first time, the localization and functioning of ABP in cell organelles – chloroplasts and mitochondria – was demonstrated at the organelles, sub-compartments and proteins

level. Formation of two different specific IAA-ABP complexes (optimum pH 5.5 and 7.5) was established in intact chloroplasts, whereas one IAA-ABP complex (pH 7.0) – in intact mitochondria. One ABP, functioning in chloroplasts (optimum pH 5.5; 50–53 kDa), was localized in stroma, another ABP (optimum pH 7.5; 38–42 kDa) – in membranes. ABP functioning in mitochondria (45–47 kDa) was localized in membranes.

It was established that IAA-ABP complexes forming in kidney bean leaf cells chloroplasts and hypocotyls cells mitochondria were different, and also they differed from other IAA-ABP complexes forming in kidney bean hypocotyls or wheat coleoptiles cells, responding to IAA by elongation growth, in cytosol and plasmalemma compartments as well as from IAA-ABP1 and IAA-TIR1 receptor complexes. The structure of IAA molecule binding sites and the function of forming IAA-ABP complexes might be different.

Basing on the available data, a presumption on possible chloroplast role in the realization of IAA hormone function was formulated.

The statements for defence:

- Auxin–binding proteins were proved to be localized and functioning in kidney bean leaf chloroplasts and hypocotyls mitochondria at the organelles, sub-compartments and proteins level.
- Intactness and functionality of organelles were significant for IAA-ABP interaction peculiarities. One IAA-ABP complex was forming in intact mitochondria, whereas two different specifically bound IAA-ABP complexes – in intact chloroplasts.
- Two ABP were functioning in intact chloroplasts: one (optimum medium pH 5.5) was localized in stroma, another (optimum medium pH 7.5) – in membranes. ABP functioning in mitochondria (optimum medium pH 7.0) were localized in membranes.
- IAA-ABP complexes forming in chloroplasts and mitochondria were different. They also differed from IAA-ABP complexes forming in kidney bean hypocotyls or wheat coleoptiles cells, responding to IAA by elongation growth, plasmalemma as well as from IAA complexes forming with ABP1 and TIR1.

Approbation of the results. The dissertation material was reported on 18 conferences: 13 international conferences and 5 conferences in Lithuania. The results of the research were presented in 9 scientific articles and in 14 abstracts of conferences.

Volume and structure of the dissertation. The doctoral dissertation is written in Lithuanian and includes an Introduction, Literature review, Research methodology, Research results, Generalization, Conclusions, References (402 cited literature sources), List of publications, 10 annexes. The dissertation is illustrated with 13 tables and 45 figures. Volume of the dissertation is 157 pages.

RESEARCH METHODOLOGY

Object of research

As the test object the sub-cellular fractions of mitochondria derived from 4-5 days old etiolated kidney bean (*Phaseolus vulgaris* L. 'Baltija') hypocotyls zone responding to IAA by elongation growth as well as chloroplasts sub-cellular fraction derived from 10-12 days old leaf were used.

Methodology of research

Isolation of chloroplasts and mitochondria sub-cellular fractions. Optimal conditions for chloroplast and mitochondria fractions isolation: composition of homogenization and rinsing medias, their pH, differential centrifugation regimes, gradients for fractions purification were chosen according to methodology used by other investigators (Кулаева и др., 1979; Fregeau, Wightman 1983; Дрейпер 1991; Селиванкина и др., 1997; Benkova et al., 1999; Lyukevich et al., 2002) and also intactness and functionality of isolated chloroplasts and mitochondria were estimated.

Protein amount in all experiments was determined by the method of Bradford (1976) or by modified (Hartree, 1972) method of Lowry (1951).

Content of chlorophyll in intact chloroplasts and their membranes fractions was determined by the method of Arnon (Arnon, 1949; Benkova et al., 1999).

Estimation of organelles intactness and functionality. The intactness and functionality of chloroplasts sub-cellular fraction was estimated by measuring oxygen evolution using Clark-type electrode (Mills, Joy, 1980) and ferricyanide photoreduction (Reeves, Hall, 1980; Николаева, 2001).

The intactness of mitochondrial fractions was assessed by measuring Cyt c oxidase (Tolbert, 1974; Benkova et al., 1999), their functionality – by measuring O_2 uptake with Clark-type oxygen electrode (Bergman et al., 1980; Journet, Douce, 1983).

Estimation of organelles sub-cellular fractions impurities. The contamination of chloroplast fraction by nucleus fragments was determined microscopically. To determine possible contamination of the chloroplast preparation by mitochondria, Cyt c oxidase, a mitochondrial marker enzyme, was assayed (Tolbert, 1974; Benkova et al., 1999).

The possible contamination of mitochondrial fractions by plasma membrane and tonoplast was determined according to ATPases activity (Gallagher, Leonard, 1982; Тихая и др., 1984; Merkys et al., 1995).

Sub-fractionation of intact organelles. For sub-fractionation of intact organelles specimens the osmotic shock procedures by three freeze (at – 70 ^{0}C) and thaw (at 4 ^{0}C)

in hypotonic medium were used (Musser, Theg, 2000; Anderson, 2001). The lysates were fractionated into stroma/matrix and membrane fractions by centrifugation: at 10 000 g for 10 min. in the case of chloroplasts and at 16000 g for 10 min. in the case of mitochondria.

Solubilization of chloroplasts and mitochondria membrane proteins. Preparations of chloroplasts membranes and mitochondria membranes vesicles (at the first stages of investigation) were solubilized, i.e. transformed to a soluble form by means of non-ionic detergent Triton X-100 (Jones, 1994). Optimal conditions were picked out experimentally. Later, mitochondrial membrane vesicles were solubilized by other non-ionic detergent Digitonin (Niznik et al., 1986; Eubel et al., 2003).

Precipitation of stroma and matrix proteins. Liquid organelles phase's soluble proteins were precipitated by ammonium sulphate (up to 80 % saturation) and purified by gel-filtration on Sephadex G-25 column.

Formation of specifically bound IAA-ABP complexes. Labeled ^{14}C-IAA concentration – 5×10^{-7} M (specific activity 1,85-2,22 GBq-mmol American Radiolabeled Chemicals, Inc.) and not labeled ^{12}C-IAR 10^{-4} M were used. To ensure the intactness and functionality of the organelles, an osmotic (sorbitol or manitol 300 mM) was added to the binding assay medium: 50mM TRIS-HCl, various pH, 1 mM KCl, 1 mM $MgCl_2$. Incubation of protein, ^{14}C-IAA, various concentrations of ^{12}C-IAA and other ligands of similar structure proceeded 30 min., at +25±2 ^{0}C temperature. The amount of bound ^{14}C-IAA was assessed in cpm (counts per minute) per 1 mg protein (protein unit). Therefore in all cases 100 cpm ^{14}C-IAA coincident 10 nM. Formation of IAA-protein complexes was applied by the method of complexes sedimentation with 10-12 % trichloroacetic acid (TCA) (Hertel at al., 1972; Ray et al., 1977 b).

Indole compounds of close chemical structure (IAA, indole-3-propionic acid (IPA), indole-3-butyric acid (IBA), indole-3-pyruvic acid (IPVA), indole-3-carboxylic acid (ICA) were tested for their ability to compete with IAA for binding sites. IAA transport inhibitors α-naphthylphtalamic acid (NPA) and 2,3,4-triiodobenzoic acid (TIBA) were also tested for their ability to compete with IAA. Concentrations used - 10^{-4} M.

Estimation of specifically bound IAA-ABP complexes characteristics. Saturation experiments were performed to estimate ^{14}C-IAA-ABP complexes binding saturation, equilibrium dissociation constant (K_D), number of binding sites (n), while using various concentrations of ^{14}C-IAA (10^{-7}-10^{-4} M) solutions. High concentration of ^{12}C-IAA (10^{-4} M) was added to the binding medium. ^{14}C-IAA-ABP complexes K_D and n were calculated by means of nonlinear regression and visualized using Scatchard plot (Scatchard, 1949; Motulsky, Christopoulos, 2003).

^{14}C-IAA displacement from specifically bound IAA-ABP complexes was estimated by the method of complexes sedimentation. ^{14}C-IAA concentration was 5×10^{-7} M and ^{12}C-IAA from 10^{-4} to 10^{-7} M. ^{12}C-IAA EC_{50} (effective concentration 50 %) was estimated by the concentration of unlabeled IAA, that blocked half of the specifically bound ^{14}C-IAA (Ray et al., 1977; Miller JR, GraphPad Software Inc., San Diego CA, 2003).

Characterization of chloroplast and mitochondria proteins. Chloroplast soluble stroma proteins both chloroplast and mitochondria membranes proteins were analyzed by nondenaturing (native) PAGE (Сафонов, Сафонова, 1969; Laemmli, 1970). ABPs native molecular masses were estimated according to the localization in nondenaturing electrophoresis gels, using Sigma standard kit (14-545 kDa), that is useful for calculating

molecular weights under neutral pH, nondenaturing conditions (Gallagher, 1999). Upper gel – 4 %, lower gel – 7,0 %.

Statistical analysis. Data were analyzed applying descriptive statistics of Microsoft Excel statistical program. Characteristics of specifically bound IAA-ABP complexes were calculated with GraphPad quickcalcs and *GraphPad Prism 4* (Miller JR, GraphPad Software Inc., San Diego CA, 2003, 30 days free trial version).

RESEARCH RESULTS AND DISCUSSION

Research on the possibilities of IAA-ABP complexes formation in sub-cellular fractions of kidney bean chloroplasts and mitochondria. To answer the question whether IAA-ABP complexes can form in chloroplasts and mitochondria, IAA and protein interaction dependence on medium pH was investigated. At the first stage, for both organelles isolation the method proposed for chloroplasts and mitochondria DNA and RNA analyse (Кулаева и др., 1979; Дрейпер 1991) using sucrose as osmotic in isolation medium was applied and purification was completed on sucrose step gradient, too. This osmotic is usually applied for purification of different cell compartments: plasmalemma, endoplasmic reticulum, tonoplast preparations for the investigation of the possibilities of IAA-ABP complexes formation (Hertel, 1979; Merkys et al., 1988; Даргинавичене, 1992; Shimomura et al., 1998; Anisimovienė et al., 2000, etc.). High purity (>90 %) chloroplast and mitochondria sub-cellular fractions were used. A possibility of chloroplasts and mitochondria interaction with IAA was analysed on binding medium pH scale from 4.0 to 9.0, every pH 0.5.

A large number of tests on binding medium pH scale demonstrated that in all sample preparations of both chloroplasts and mitochondria total ^{14}C-IAA binding was ascertained. The obtained data allowed us to presume about a possible functioning of ABP forming specific complexes with IAA in chloroplasts at optimum pH 5.5 and in mitochondria – optimum pH 7.0. Mean values of the test data demonstrating a possibility of IAA-ABP interaction are presented on the 1 table. However, a constant recurrence of the results was not obtained.

Thus, it was doubtful whether organelles' intactness and functionality was ensured when sucrose as osmotic was used in fractions isolation medium and their purification was carried out on sucrose step gradient. This presumption was supported by the opinion of other investigators (Moppe et al., 1987) that chloroplasts, which are purified on sucrose gradient, may almost fully lose their ability to fix CO_2.

Table 1. Estimation of optimal pH for IAA-ABP complexes formation in organelles sub-cellular fractions

Organelle	Binding medium pH	Total ^{14}C-IAA binding, cpm./1 mg protein; M±SE	Specific ^{14}C-IAA binding, cpm./1 mg protein; M±SE	IAA-ABP complexes specificity, %; M±SE
Chloroplasts	5.5	4247.3±115.2	1783.3±78.3	41.9±1.8
	6.0	3763.5±34.8	419.3±45.3	11.1±0.1
Mitochondria	7.0	14763.3±796.3	3212.7±196.4	21.8±1.3
	7.5	14221.3±729.3	853.3±98.7	5.9±2.2
	8.0	10060.8±642.4	507.0±46.4	5.0±0.1

To ground or reject this presumption, intactness and functionality of chloroplasts and mitochondria sub-cellular fractions was evaluated: a) fractions isolated by using sucrose as osmotic and purified on sucrose step gradient, b) fractions isolated by using sorbitol (in case of chloroplasts) and manitol (in case of mitochondria) as osmotic and purified on percoll step gradient (Fregeau, Wightman, 1983; Lyukevich et al., 2002).

Table 2. Comparison of isolation conditions influence on intactness and functionality of chloroplasts sub-cellular fractions

Characteristic	Unpurified chloroplasts fraction	Chloroplasts fraction purified on sucrose gradient	Chloroplasts fraction purified on percoll gradient
Intactness, %; M±SE *	48.8±1.4	72.5±3.0	91.5±3.1
O_2 evolution, μM/mg chl/h; M±SE	54.9±2.7	78.8±3.1	113.0±6.1
$K_3Fe(CN)_6$ photoreduction, mM/mg chl/h; M±SE	369.8±13.8	590.2±20.5	720.3±24.1

* - according both measurements indexes average

The results obtained by measuring (Table 2) chloroplast fraction O_2 evolution during photosynthesis (Mills, Joy, 1980) and ferricyanide photoreduction (Reeves, Hall, 1980) as well as mitochondria sub-cellular fractions (Table 3) – by measuring O_2 uptake (Journet, Douce, 1983) and cytochrome c (Cyt c) oxidation (Tolbert, 1974) demonstrated that: a) chloroplasts sub-cellular fractions isolated by using sorbitol as osmotic and purified on percoll step gradient, b) mitochondria sub-cellular fractions, isolated by using manitol as osmotic and purified on percoll gradient, distinguish in high intactness as well as functionality, therefore, they can be used for the research on IAA-ABP complexes

formation in organelles. The above-discussed data correlate with the results discovered in literature references (Jackson et al., 1979; Mills Joy, 1980).

Table 3. Comparison of intactness of mitochondria sub-cellular fractions unpurified and purified on percoll gradient

Characteristic	Unpurified mitochondria fraction	Percoll-purified mitochondria fraction
Mitochondria fraction Cyt c oxidized nM/min./1 mg protein; M±SE	13.5±1.4	5.1±0.5
Mitochondria fraction disrupted by Triton X-100 Cyt c oxidized nM/min./1 mg protein; M±SE	192.1±2.0	198.8±3.8
Intactness, %; M±SE	92.9±1.2	97.4±1.1

It was also established that in ^{14}C-IAA binding medium with osmotic (sorbitol or manitol, respectively) intact chloroplasts and mitochondria better retain functionality for exposition with ^{14}C-IAA for a necessary period (30 min.), thus, the research on IAA-ABP complexes formation in intact organelles was carried out by using the medium with osmotic.

IAA-ABP complexes formation in intact and functional preparations of kidney bean chloroplasts and mitochondria

The research on a possible ^{14}C-IAA interaction with chloroplasts ABP, when intactness and functionality of organelles were ensured, proved both the formation of specific IAA-ABP complexes at optimum pH 5.5 and presence of the second IAA binding site at optimum pH 7.5 (Fig. 1 A).

The second IAA binding site (optimum pH 7.5) in intact chloroplast preparations showed specific binding activity 1.6 fold lower (3358.0±302.6 ^{14}C-IAA cpm/1 mg protein) than the site functioning at pH 5.5 (5254.2±168.3 ^{14}C-IAA cpm/1 mg protein). The specificity of these IAA-ABP complexes was also lower (Fig. 1 A), correspondingly 51.0±2.9 % (pH 5.5) and 43.2±3.9 % (pH 7.5). However, at both binding media pH (5.5 and 7.5), intensive specific IAA and ABP interaction was determined, therefore, in further investigations on IAA-ABP complexes formation in chloroplasts sub-cellular fraction, ^{14}C-IAA and ABP interaction at both pH (5.5 and 7.5) was analysed.

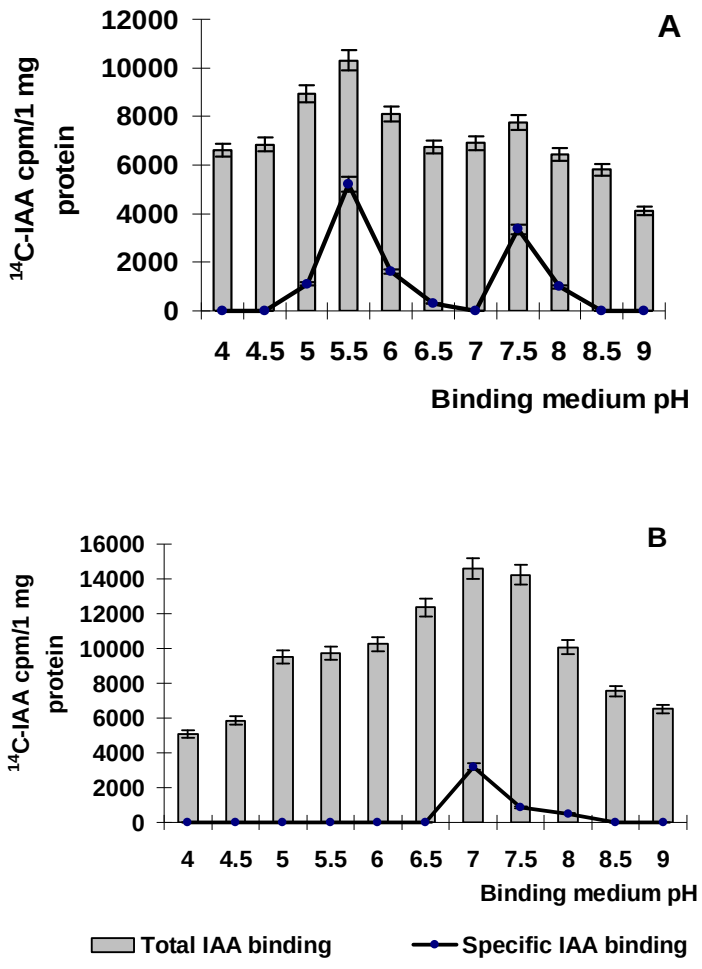


Fig 1. Dependence of IAA and ABPs interaction in intact chloroplasts (A) and mitochondria (B) organelles fractions on medium pH.

From the available literature references it is clear that in other cell compartments not single IAA site can also function: in wheat coleoptiles plasmalemma at optimum pH 5.5 and 7.2 (Merkys et al., 1988; Даргинавичене, 1992), maize coleoptiles plasmalemma – pH 5.5 (Ray et al., 1977 b; Hertel, 1979; Nakamura, Ono, 1988, etc.) and 7.0 (Nave, Benveniste, 1984), kidney bean hypocotyls plasmalemma – pH 5.5 and 7.5 (Anisimovienė, Jodinskienė, 1999; Jodinskienė, 2005), wheat coleoptiles tonoplast – pH 5.5 and 8.0 (Merkys et al., 1998; Darginavičienė, Novickienė, 2002).

Intactness and functionality of mitochondria organelles, contrary to that of chloroplasts, did not reveal new IAA binding sites. Optimal specific IAA binding and formation of specific IAA-ABP complexes was ascertained at pH 7.0 (Fig. 1 B). In that case, the amount of specifically bound ^{14}C-IAA was 8346.4±435.9 cpm/1 mg protein, whereas specificity of complexes came to 41.3±3.6 %. Assurance of intactness and functionality of mitochondria organelles had significant influence upon ABP and IAA interaction: total amount of associated IAA with 1 mg protein was 1.3 fold higher, specific IAA binding was 2.5 fold higher, whereas the specificity of complexes augmented by 20 % in comparison with the cases when fractions were isolated and purified by using sucrose as osmotic.

Characterization of forming IAA-ABP complexes. To estimate the characteristics (concentration of IAA ligand for ABPs site saturation, K_D, n) of specific IAA-ABP complexes forming in chloroplasts and mitochondria, a specific binding

dependence upon ^{14}C-IAA concentration in the medium was analysed. It was determined that intact chloroplasts' ABP at optimum medium pH 5.5 tended towards saturation with ^{14}C-IAA ligand when its concentration reached 8.5-9×10^{-6} M (Fig. 2 A).

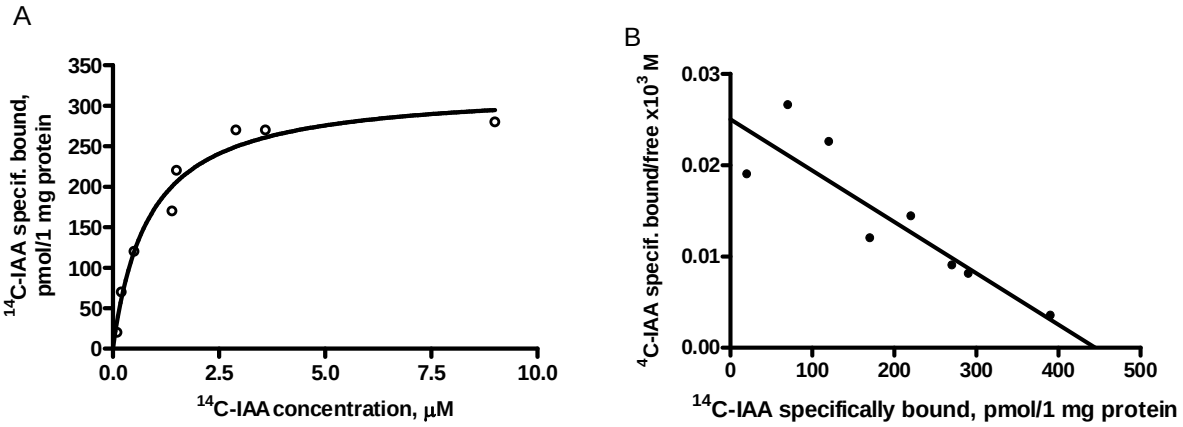


Fig. 2. Dependence of specific interaction between IAA and intact chloroplasts proteins on ^{14}C-IAA concentration at medium of pH 5.5 (A) and Scatchard plot of the same dependence (B).

^{14}C-IAA binding data analysis in the standard Scatchard plot (Fig. 2 B) indicated that IAA-ABP complexes had a K_D = 1.14±0.51×10^{-5} M, n = 2.00±0.63 nmol/1 mg protein or correspondingly 16.30±1.59 nmol/1 g fresh weight.

The second ABP saturation with ^{14}C-IAA ligand in intact chloroplasts at optimum medium pH 7.5 was almost completed at its concentration 8-9×10^{-5} M (Fig. 3 A). Under those conditions, the IAA-ABP complexes equilibrium dissociation constant was K_D = 1.72±0.59×10^{-5} M, the number of binding sites n = 0.44±0.02 nmol/1 mg protein or 3.47±0.02 nmol/1 g fresh weight (Fig. 3 B).

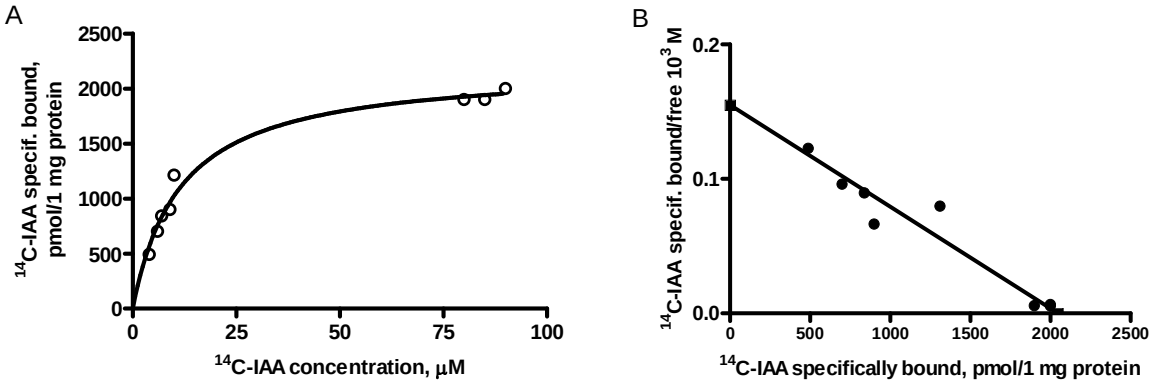


Fig. 3. Dependence of specific interaction between IAA and intact chloroplasts proteins on ^{14}C-IAA concentration at medium of pH 7.5 (A) and Scatchard plot of the same dependence (B).

ABP saturation with IAA in intact mitochondria at pH 7.0 can be reached only at ^{14}C-IAA concentrations not lower than 4-5×10^{-4} M (Fig. 4 A). The equilibrium dissociation constant of the forming IAA-ABP complexes was 1.90±0.51×10^{-5} M, n = 3.85±0.34 nmol/1 mg protein or 11.48±1.01 nmol/1 g fresh weight, respectively (Fig. 4 B).

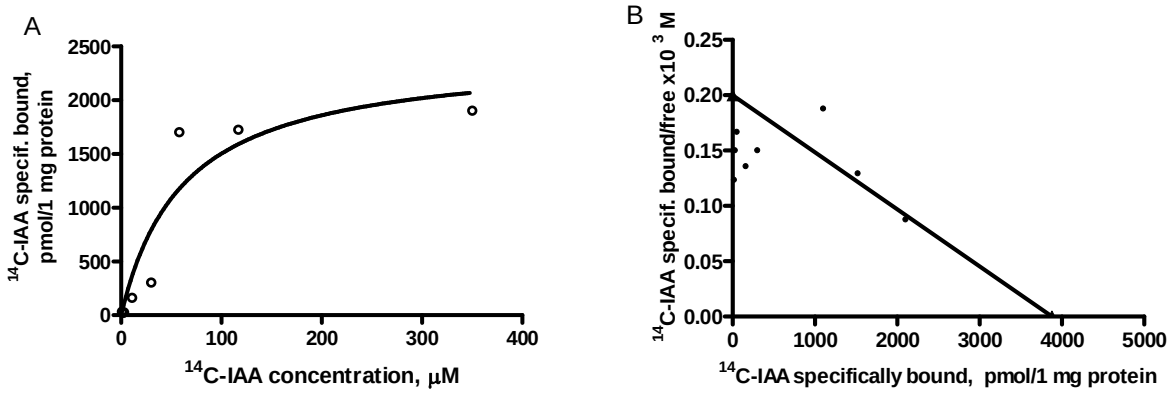


Fig. 4. Dependence of specific interaction between IAA and intact mitochondria proteins on ^{14}C-IAA concentration at medium of pH 7.0 (A) and Scatchard plot of the same dependence (B).

The research on ^{14}C-IAA displacement from IAA-protein complexes enabled to estimate ^{12}C-IAA concentration that blocked 50 % of the specifically bound ^{14}C-IAA or EC_{50} (effective concentration). In intact chloroplasts' organelles at pH 5.5 it was 1.04±0.13×10^{-5} M, at pH 7.5 – 1.62±0.15×10^{-5} M, in intact mitochondria organelles at pH 7.0 – 1.98±0.19×10^{-5} M.

As far as there is no information available on the complexes forming in those organelles as well as their characteristics, it was possible to compare them only with the characteristics of IAA-ABP complexes forming in other cell compartments. Higher IAA ligand concentrations are necessary for the saturation of ABP functioning in intact chloroplasts and mitochondria, ABP has lower affinity for IAA ligand, whereas the number of binding sites is larger than in IAA-ABP complexes forming in kidney bean hypocotyls plasmalemma (Jodinskienė, 2005; Jodinskienė, Anisimovienė, 2006), maize plasmalemma (Ray et al., 1977 b; Nave, Benveniste, 1984) as well as wheat coleoptiles cells plasmalemma (Даргинавичене, 1992) at the same pH.

Competitive ability of other ligands for IAA binding sites in the preparations of intact chloroplasts and mitochondria. It was estimated how indole compounds of close chemical structure displace IAA from specific complexes forming in the preparations of kidney bean leaf intact chloroplasts and kidney bean hypocotyls intact

mitochondria. The obtained results (Fig. 5) showed that ^{12}C-IAA had the highest competitive ability for ^{14}C-IAA binding sites in ABP molecule in both intact chloroplast (at pH 5.5 – 48.1±4.6 % and pH 7.5 – 40.5±3.9 %) and intact mitochondria (at pH 7.0 – 38.3±4.2 %) preparations.

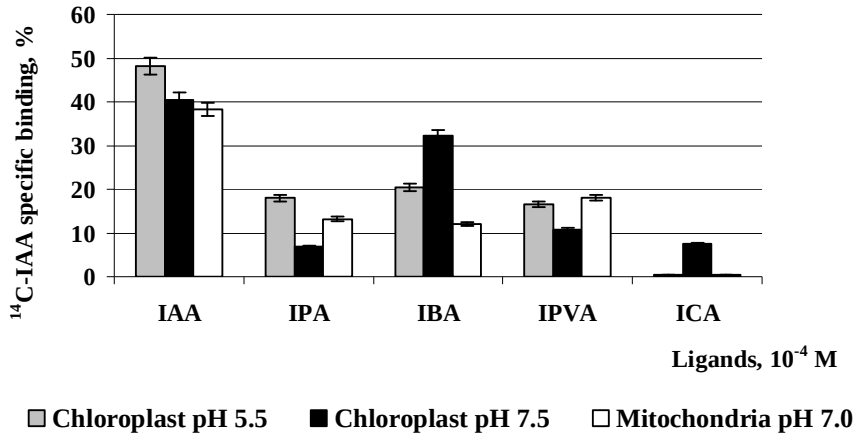


Fig. 5. Comparison of close structure indole compounds in competing for ^{14}C-IAA binding sites.

IBA ligand actively competed for ^{14}C-IAA binding to ABP sites in intact chloroplasts at both pH media – 20.4±2.3 % and 32.2±3.4 %, respectively (Fig. 5), whereas IPA and IPVA ligands a little more actively competed only at pH 5.5 (18.0±2.6 % and 16.5±1.8 %), and at pH 7.5, ^{14}C-IAA displacement was weak, only margin of error. In the fraction of intact mitochondria, IPVA demonstrated the most active ^{14}C-IAA displacement – 18.1±1.6 %, however, it was twice lower than that of ^{12}C-IAA (Fig. 5). IPA and IBA competed similarly – 13.2±1.2 % and 12.0±0.7 %, respectively.

Physiologically inactive IAA destruction product – ICA showed low competitive ability or did not compete for IAA binding sites neither in IAA-ABP complexes of intact chloroplasts or those of mitochondria (Fig. 5).

From the available data, which show that physiologically active compounds compete, whereas physiologically inactive compound ICA (product of IAA decarboxilation) does not compete for^{14}C-IAA binding sites, it is possible to presume that the interaction between in those organelles localized ABP and IAA may have biological significance. The obtained data coinsident with data of other investigators obtained in researching of ligands' competitive ability for IAA binding sites in other cell compartments: maize

coleoptiles membranes (Ray et al., 1977 b), kidney bean leaf membranes (Wardrop, Polya, 1980), wheat coleoptiles plasmalemma (Даргинавичене, 1992).

Influence of IAA transport inhibitors on IAA-ABP complexes formation in the preparations of intact chloroplasts and mitochondria. For a more detailed characterization of specific IAA-ABP complexes forming in chloroplast and mitochondria organelles, the influence of IAA transport inhibitors TIBA and NPA was assessed.

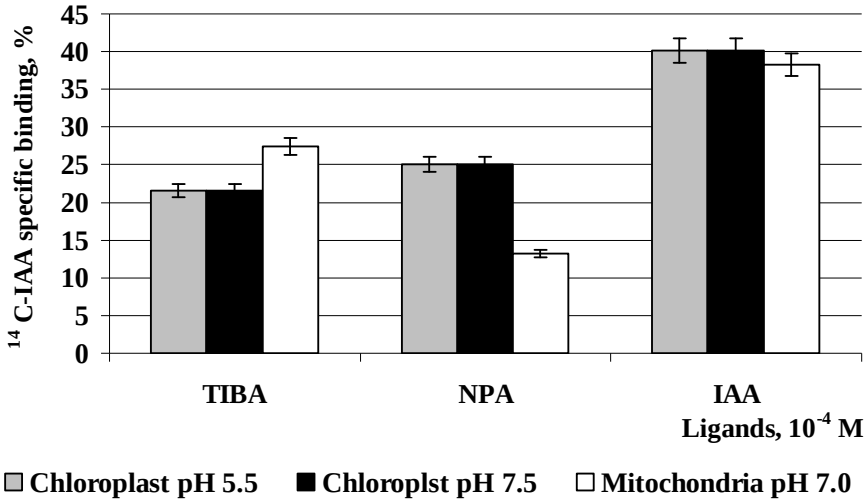


Fig. 6. Peculiarities of IAA transport inhibitors competing for ^{14}C-IAA binding sites in intact chloroplasts and intact mitochondria fractions.

TIBA actively competed for all in both investigated organelles IAA binding to ABP sites: in intact chloroplast fraction it displaced ^{14}C-IAA from binding sites by 29.1±2.5 % (at optimum pH 5.5) and 21.5±1.8 % (pH 7.5), in the fraction of mitochondria – 27.4±3.7 % (pH 7.0).

Another transport inhibitor NPA demonstrated unlike competitive ability for IAA-ABP binding sites in those organelles. In intact chloroplast fraction it competed actively (25.0±2.3 %) at close to neutral medium pH 7.5, in mitochondria fraction at neutral medium pH 7.0 the displacement of ^{14}C-IAA was twice lower (13.2±1.6 %), whereas at acid medium in chloroplast fraction it practically did not compete (Fig. 6).

The comparison of TIBA competitive ability for IAA-ABP complexes forming in kidney bean hypocotyls plasmalemma at medium pH 5.5 and 7.5 (Jodinskienė, 2005) allowed to ascertain analogous activity – 25.8 % and 27.2 %, respectively. Other investigators also demonstrated that TIBA intensively competed for auxin NPA binding

sites at pH 5.5, too (Batt et al., 1976; Wardrop, Polya, 1980; Murphy, 1980; Löbler, Klämbt, 1985).

IAA transport inhibitors TIBA and NPA were shown to inhibit, both *in vivo* and *in vitro*, the processes of specific IAA binding with proteins at medium pH 7.2 in plasmalemma vesicles isolated from wheat coleoptiles cells (Darginavičienė, Maksimov, 2001). In kidney bean hypocotyls plasmalemma of the same studied plant, ^{12}C-NPA competitive ability for ^{14}C-IAA binding to ABP was low – 5.8 % at pH 7.5 and 2.8 % at pH 5.5.

Compartmentation of auxin recognizing proteins in organelles. To confirm or reject a possibility that several different ABP can function in plant cell organelles – chloroplasts and mitochondria as well as that interaction between ABP and IAA localized in these organelles may have biological significance, more detailed investigations were necessary. First of all, we carried out the experiments allowing us to determine in which sub-compartment of the organelles were localized and functioning the IAA recognizing proteins, forming specific complexes with IAA. For that purpose, chloroplasts and mitochondria were disrupted by osmotic shock and a possibility of IAA-ABP complexes formation was analysed in the fraction of all membranes of the organelle and in the fractions of its liquid phases (stroma or matrix).

IAA-ABP complexes formation in kidney bean chloroplasts sub-compartments – membranes and stroma. Possibility of IAA-ABP complexes formation in chloroplasts' sub-compartments– membrane vesicles and stroma fractions was also analysed at binding medium pH scale from 4.0 to 9.0. The curves obtained after the analysis of specific ^{14}C-IAA and ABP binding as well as specificity of formed complexes showed that

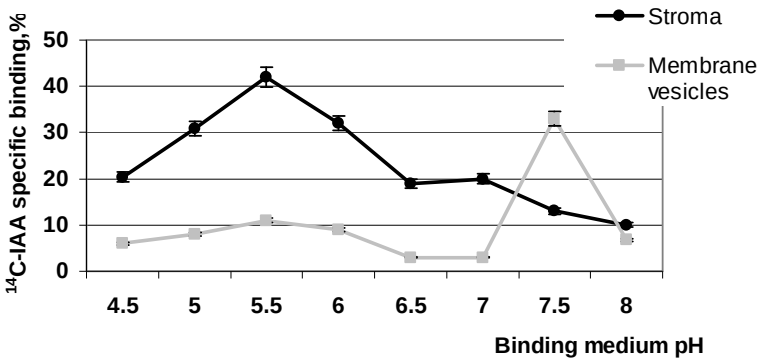


Fig. 7. Dependence of IAA-ABP complexes formation in different chloroplasts sub-compartments on binding medium pH.

each of both analysed chloroplasts' sub-compartments had one different peak of specific IAA binding to ABP (Fig. 7).

In the preparations of stroma, specifically bound IAA-ABP complexes were forming at binding medium pH 5.5 (Fig. 7). Specificity of forming complexes was 42.3±2.0 %; the amount of ^{14}C-IAA binding activity was 3936.3±189.0 cpm/1 mg protein. In the preparations of membrane vesicles, optimal IAA-ABP complexes formation was observed at medium pH 7.5. Specificity of forming complexes was 32.9±1.3 % (Fig. 7).

Research on IAA-ABP complexes formation in mitochondria sub-compartments – membranes and matrix. To determine in which sub-compartment (membranes or matrix) of mitochondria IAA binding proteins were localized, the experiments on auxin and proteins interaction in both sub-compartments of mitochondria were performed at binding medium pH 7.0.

The results showed that in mitochondria matrix fraction, the interaction with IAA was very weak, which could not be estimated as specific. That was also confirmed by the tests in the whole pH scale from 4.0 to 9.0 as well as by the IAA binding experiments employing specimens of mitochondria sub-compartment proteins precipitated by ammonium sulphate up to 80 % saturation.

IAA and ABP interaction in the preparations of mitochondria membrane vesicles was twice more active than that in matrix fraction. The amount of specifically bound ^{14}C-IAA reached 390.9±12.0 cpm/1 mg protein. Specificity of formed IAA-ABP complexes was 9.3±1.7 % (data present in next chapter Table 5). Possible ABP localization in mitochondria membrane sub-compartment was also confirmed by the data obtained by performing experiments on the possibility of ^{14}C-IAA and ABP interaction in mitochondria membrane vesicles within the whole binding medium pH scale as well as with membrane fractions obtained by applying another methods for mitochondria membrane isolation, which ensures integrity of membrane structures (Hájek et al., 2004).

Characterization of auxin and protein interactions in different sub-compartments of chloroplasts and mitochondria

IAA-ABP complexes formation in chloroplasts stroma. The comparative experiments on IAA-ABP complexes forming in stroma sub-compartments fraction and stroma protein preparations demonstrated that stroma proteins' precipitation by ammonium sulphate and purification on Sephadex G–25 columns did not affect on

protein ability to specifically interact with IAA. Besides, the purified stroma protein preparations specifically bound labelled IAA by 20 % more – 4732.9±265.9 cpm/1 mg protein. Specificity of formed IAA and purified stroma protein complexes was even by 15 % higher than that of IAA and native stroma proteins. It reached 49.2±3.1 %, which corresponded to the specificity of complexes forming in intact and functional chloroplasts (51.0±2.9 %) at the same pH.

IAA-ABP complexes formation in chloroplasts membranes. Preparations of chloroplast membrane vesicles were solubilized, i.e. proteins were transformed to a soluble form by means of non–ionic detergent Triton X–100. Protein–detergent micelles were purified by Amberlit XAD–4 to eliminate the excess of Triton X–100. The results of investigations revealed that solubilized chloroplast membrane proteins maintained their ability to recognize and specifically bind to IAA, and the amount of specifically bound ^{14}C-IAA per protein unit was 1.5 fold higher than in membrane vesicles preparations and correspondingly made 3727.5±257.9 and 2485.9±167.8. Specificity of formed complexes reached 36.4±2.0 %.

Therefore, to specify and compare chloroplast liquid phase (stroma) as well as membrane ABP and phytohormone interactions with the peculiarities of functioning in other cell compartments isolated ABP and phytohormone interactions, the main characteristics of complexes (K_D, n, ligand as well as transport inhibitors ability to compete for IAA binding sites) were repeatedly estimated by using the preparations of solubilized membrane proteins and stroma proteins.

The data (Table 4) demonstrated that both stroma proteins and solubilized membrane proteins affinity for IAA ligand was higher than that of intact chloroplast proteins at the same pH values.

The above–discussed property correlates with the obtained characteristics of auxin receptor ABP1 when investigating the interaction of auxin NAA and this protein in plasmalemma membrane preparations (Löbler, Klämbt, 1985 a) and solubilized as well as purified ABP1 (Cross, Briggs, 1978; Löbler, Klämbt, 1985 a; Jones, 1994). The number of IAA binding to ABP sites in both sub-compartments decreased by one order in comparison with intact organelle. Besides, the number of solubilized chloroplast membrane proteins was smaller by one order than that of IAA binding to stroma ABP.

Table 4. Comparison of main characteristics of IAA-ABP complexes formed in chloroplasts and mitochondria of kidney bean cells

Compartment	Characteristics of IAA-ABPs				
	pH	Complexes specificity, %	K_D, M	Number of binding sites mol/1 mg protein	ABP molecular mass
Chloroplasts stroma	5.5	49.2±3.1	$4.62±0.42×10^{-6}$	$1.30±0.28×10^{-10}$	50-53
Cloroplasts membranes	7.5	36.4±2.1	$1.14±0.39×10^{-6}$	$3.08±0.40×10^{-11}$	38-42
Mitochondria membranes	7.0	34.2±2.4	$8.49±0.51×10^{-5}$	$5.29±0.72×10^{-9}$	45-47

Assessment of indole compounds of close chemical structure, the same as in intact organelles, ability to displace ^{14}C-IAA from complexes forming with purified stroma proteins and solubilized membrane proteins (Fig. 8) showed that although the activity of some ligands slightly varied, the order was analogous to intact and functional chloroplast cases at corresponding medium pH values.

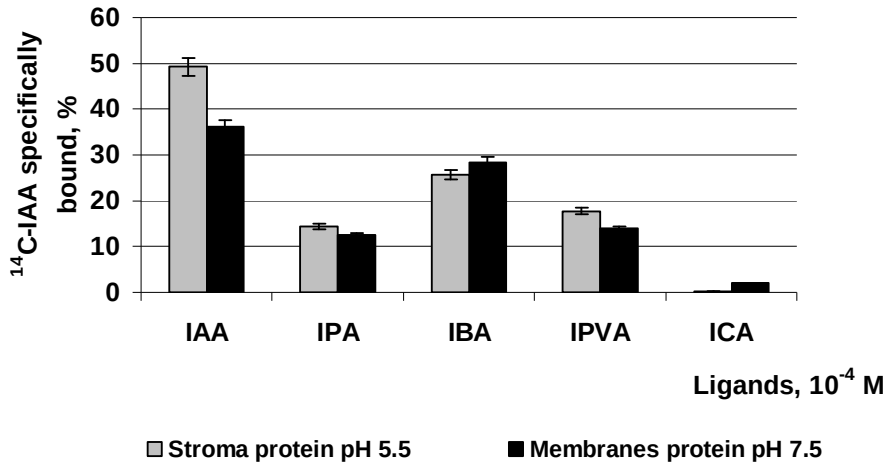


Fig. 8. Comparison of close structure indole compounds in competing for ^{14}C-IAA binding with precipitated chloroplast stroma proteins and solubilized membrane proteins sites.

Estimation of IAA transport inhibitors ability to displace ^{14}C-IAA from IAA complexes with solubilized chloroplasts membrane proteins revealed that in this case TIBA demonstrated more active competitive ability (18.3±1.6 %) in comparison with intact organelles.

IAA-ABP complexes formation in mitochondria membranes For a more detailed characterization of IAA interaction with mitochondria membrane proteins were

solubilized at first by means of non–ionic detergent Triton X–100. In order to solubilize proteins capable to interact with IAA from mitochondria membrane vesicles, the optimal conditions for solubilization by this detergent were selected experimentally (Table 5) by applying detergent concentration (from 0.5 to 2.0 %) as well as the most proper ratio between protein and non–ionic detergent Triton X–100 (from 1:1 to 1:5).

Table 5. Comparison of IAA-ABP complexes forming in mitochondria

Object	Specific ^{14}C-IAA binding cpm/1 mg protein	IAA-ABP complexes specificity, %
Intact mitochondria	8346.4±43.9	41.3±3.6
Mitochondria membrane vesicles	5716.0±390.0	12.5±0.6
Mitochondria membrane protein solubilized with Triton X-100*	6254.2±361.1	14.8±0.8
Mitochondria membrane protein solubilized with Digitonin**	7499.3±482.5	34.2±2.4

*Triton X-100 concentration 1.75 %, ratio of protein:detergent 1:3
**Digitonin concentration 1.0 % ratio of protein:detergent 1:5

However, the results (Table 5) showed that this detergent did not completely solubilize proteins or the protein, which recognized and interacted with IAA in mitochondria membranes. Wherefore, detergent Triton X–100 was replaced by another non–ionic detergent – digitonin applied namely for the destruction of mitochondria, lipoprotein complexes (Nizmik et al., 1986; Eubel et al., 2003).

Solubilized by this detergent mitochondria membrane protein and IAA interaction was more intensive – the amount of ^{14}C-IAA per protein unit was 7499.3±482.5, whereas the specificity of formed complexes significantly increased – 34.2±2.4 % (Table 5). Both specific binding and specificity of forming complexes were shown to be similar to ABP and IAA interaction in intact mitochondria organelles.

Analysis of IAA interaction with mitochondria membrane proteins solubilized by digitonin at medium pH 7.0 revealed that protein affinity for IAA ligand, as in chloroplast stroma and membrane cases, increased insignificantly compared with intact organelles ABP affinity for ligand at the same medium pH (Table 4 present in previous chapter). The same order was retained – $8.49\pm0.51\times10^{-5}$ M. The number of ^{14}C-IAA binding sites ($5.29\pm0.72\times10^{-9}$ mol/1 mg protein) compared with intact ($3.85\pm0.34\times10^{-9}$ mol/1 mg protein) decreased insignificantly as in chloroplast case, it was by one order

higher than the number of IAA binding sites to stroma ABP and even by two orders higher than that of IAA binding sites to ABP localized in chloroplast membranes. Therefore, the research data revealed that the solubilized mitochondria membrane proteins affinity for IAA was lower than that of chloroplast stroma and membranes ABP.

As in intact mitochondria organelle preparations, ^{12}C-IAA possessed the highest competitive ability for ^{14}C-IAA in solubilized mitochondria proteins, also. Activity of other ligands was as follows: IAA>IPVA>IBA>IPA. Inactive compound ICA did not compete for IAA binding sites like in intact mitochondria organelles.

Analysis of IAA transport inhibitors ability to displace ^{14}C-IAA from IAA-ABP complexes with solubilized mitochondria membrane proteins enabled to determine that in this case both ligands displaced ^{14}C-IAA from binding sites in the same manner as in intact mitochondria organelles.

Therefore, the research on ABP at intact mitochondria and chloroplast organelles and their sub-compartment levels revealed that three sites were functioning in those organelles: two in membranes (chloroplasts and mitochondria) and one in chloroplast stroma.

Identification of auxin binding proteins (ABP) functioning in organelles. Preparations of precipitated chloroplast stroma as well as solubilized chloroplasts and mitochondria membrane proteins were analysed by native (non-denaturing) PAGE method. Non-stained gels were cut into zones (as shown in Fig. 9 B, C, D). Proteins were extracted from each zone with 50 mM TRIS-HCL buffer at corresponding pH and their capacity to specifically bind ^{14}C-IAA was examined to detect the zone of proteins capable to recognize IAA.

Specifically bound IAA-ABP complexes forming in chloroplasts stroma. 14 protein components of different electrophoretic mobility were detected in the purified chloroplast stroma protein preparation (Fig. 9 B).

Analysing proteins extracted from three gel zones (Fig. 9 B, shown as I, II, III) with 50 mM TRIS-HCl buffer at pH 5.5, we detected the significant specific IAA binding only in the middle zone (II) of relative electrophoretic mobility (REM) (Fig. 10 A) – 3428.2±301.2 ^{14}C-IAA cpm/1 mg protein. Specificity of the formed complexes reached to 33.0±2.5 %.

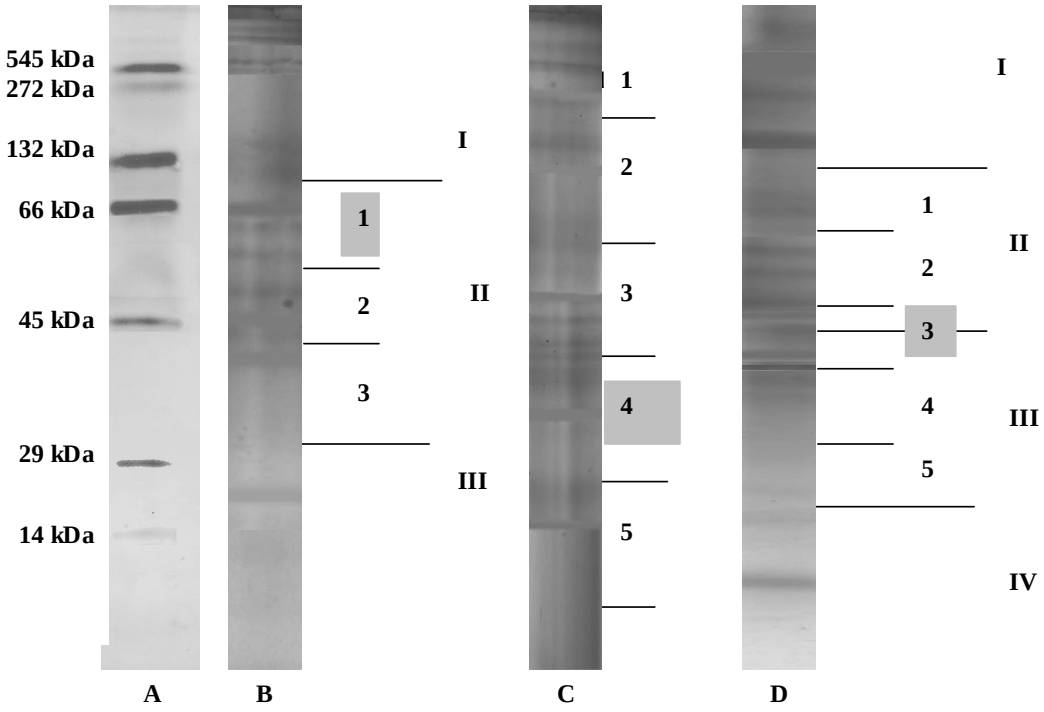


Fig. 9. Electroforegrams of protein standards **(A)**, chloroplast stroma **(B),** chloroplast membrane **(C),** mitochondria membrane (**D)** proteins.

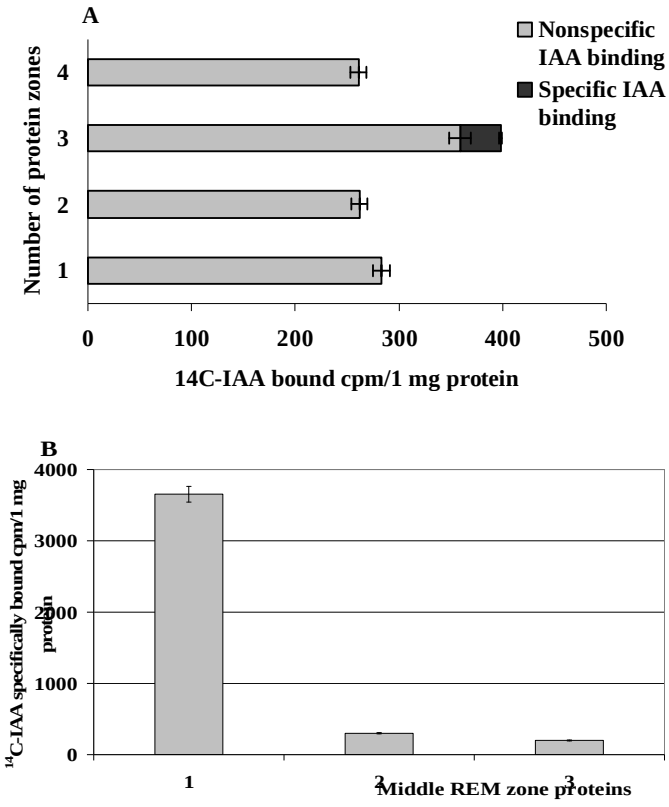


Fig. 10. Capability of chloroplasts stroma proteins localized in different zones of gels to bind ^{14}C-IAA (**A**) and ^{14}C-IAA binding in separate parts of middle zone (**B**).

At the following stages, zone II was cut into three parts (Fig. 9 B, shown as 1, 2, 3) according to protein localization place identified after staining of several pathways of the same gel. Analysis of the ability of proteins extracted from these zones to interact with ^{14}C-IAA (Fig. 10 B) showed that specific IAA binding was characteristic of the zone 1th with 2 proteins. The amount of ^{14}C-IAA specifically bound per 1 mg protein was 3654.8±375.6 cpm, the specificity of formed complexes – 37.6±2.9 %.

The obtained results enabled to conclude that molecular mass of proteins interacting with IAA in stroma could range from 50 to 53 kDa (Fig. 9 B).

Specifically bound IAA-ABP complexes forming in chloroplasts. 11 proteins of different molecular mass were electrophoretically detected in the preparations of solubilized chloroplast membrane proteins (Fig. 9 C).

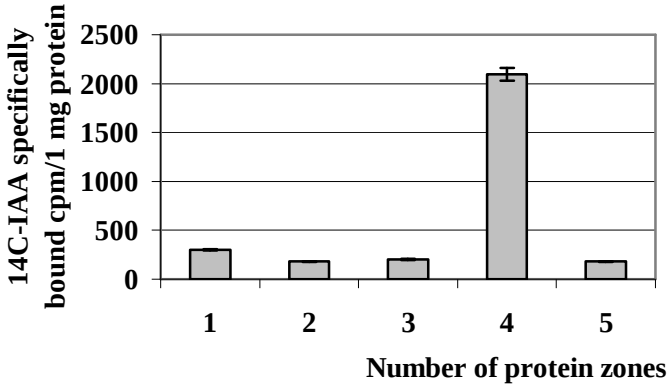
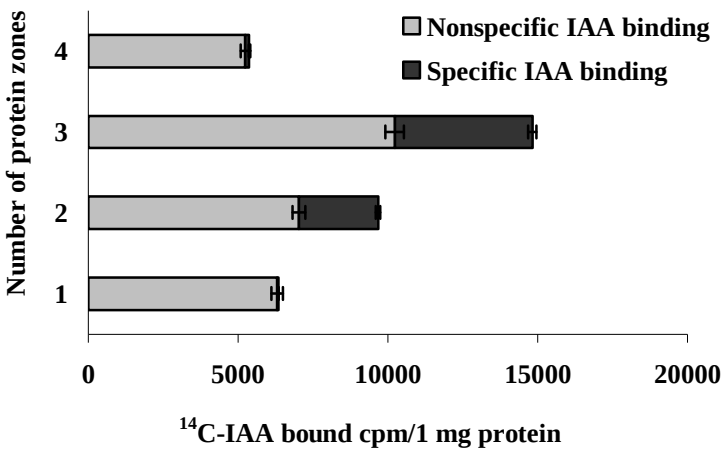


Fig. 11. Capability of chloroplast membrane protein zones with different mobility to bind ^{14}C-IAA.

Proteins from 5 gel zones (Fig. 9 C) were extracted with 50 mM TRIS-HCl buffer at pH 7.5. Specific ^{14}C-IAA binding was ascertained only in the 4th zone – 2095.6±206.2 cpm/1 mg protein, specificity of formed complexes – 34.2±2.2 % (Fig. 11).

In comparison with standard localization places in the same gels (Fig. 9 C), we may conclude that molecular mass of proteins interacting with IAA in chloroplast membranes ranged between 38 and 42 kDa.



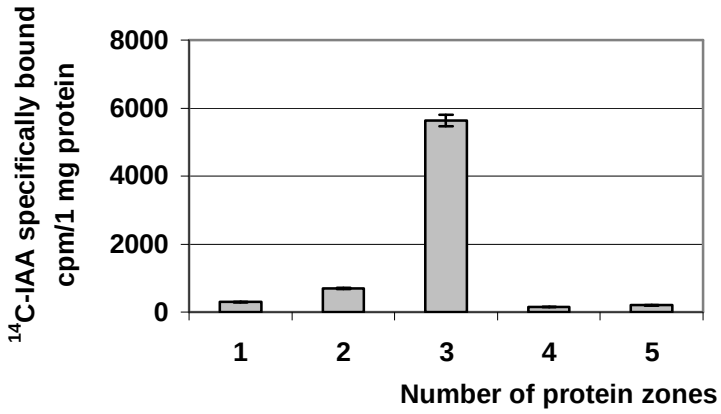


Fig. 12. Capability of mitochondia proteins with different mobility (**A**) and middle REM zone (**B**) proteins to bind ^{14}C-IAA.

Specifically bound IAA-ABP complexes forming in mitochondria membranes. 16 protein zones (Fig. 9 D) were detected in electrophoretic gels of solubilized mitochondria membrane protein preparations. Proteins were extracted with 50 mM TRIS-HCl buffer at pH 7.0 from 4 gel zones (Fig. 9 D, shown

as I, II, III, IV). Significant specific IAA binding was established in the 2nd and 3rd zones (Fig. 12 A). In the 2nd zone, the amount of ^{14}C-IAA specifically bound per 1 mg protein was 2650.1±209.6 cpm, specificity of formed complexes – 18.5±1.2 %. In the 3rd zone, the specific interaction of extracted proteins was 4596.0±501.4 ^{14}C-IAA cpm/1 mg protein, specificity of formed IAA-ABP complexes – 31.4±2.9 % (Fig.12 B).
Further, this electrophoretic zone was cut into 5 parts (Fig. 9 D, shown as 1–5). Specific IAA binding was characteristic of the 3rd zone. The amount of ^{14}C-IAA specifically bound per 1 mg protein was 5641.6±553.6 cpm, whereas specificity of formed IAA-ABP complexes – 40.9±3.5 %.

The molecular mass of proteins interacting with IAA in mitochondria membranes ranged from 45 to 47 kDa (Fig. 9 D).

Thus, the localization and functioning of specific ABP was demonstrated at three levels: intact organelles, their sub-compartments and proteins. The obtained results showed that one IAA binding site was functioning in mitochondria organelles, and two sites – in chloroplast organelles. Although both ABP (soluble and membrane) in chloroplast and one ABP (membrane) in mitochondria were proteins of low molecular mass, however, the characteristics of their interaction with IAA (specificity of complexes, amount of IAA bound to protein unit, K_D, EC_{50}, n, ABP saturation with IAA, etc.) significantly differed. The characteristics of ABP functioning in those organelles notably differed from the characteristics of other ABP detected in kidney bean elongating hypocotyls cells (Table 6) – two in plasmalemma (Anisimovienė et al., 2000; Merkys et al., 2004; Jodinskienė, 2005; Jodinskienė, Anisimovienė, 2006) and two in cytosol (Mockevičiūtė, Anisimovienė, 1999; Anisimovienė et al., 2000).

They also differed from the characteristics (Table 6) of commonly acknowledged auxin receptors: ABP1 – functioning in plasmalemma (Löbler, Klämbt, 1985, Jones, 1994) and TIR1 – functioning in nucleus (Dharmasiri et al., 2005 a; Kepinski, Layser, 2005) as well as from IAA-protein complexes forming in plasmalemma and tonoplast compartments from wheat coleoptiles cells responding to IAA by elongation (Merkys et al., 1988; Даргинавичене, 1992; Darginavičienė, Novickienė, 2002). The obtained results lead to presumption that IAA-ABP functions may be different than those of IAA-receptor complexes in plasmalemma and nucleus.

Table 6. Comparison of main characteristic of IAA-ABP complexes identified in various plant cell compartments

Cell compartment	Characteristics			Known or putative function	Authors, year
	pH	K_D, M	ABP mol. mass		
ABP1 maise plasmalemma	5.5	IAR – 3.9×10^{-6} NAR 3-6×10^{-8}	40 (2×22)	Receptor	Löbler et al., 1985; Jones, 1994
TIR1 arabidopsis nuclear	7.5 7.2	2.5×10^{-8} 8.4×10^{-8}	?	Receptor	Kepinski, Leyser, 2005; Dharmasiri et al., 2005a
ABP wheat plasmalemma	5.5 7.2	1.1×10^{-8} 3.0×10^{-8}	20 80-90	Receptor?	Даргинавичене, 1992
ABP kidney bean plasmalemma	5.5 7.5	$1.65\pm0.54\times10^{-7}$ $5.86\pm0.68\times10^{-7}$	26 29-45	Receptor Transporter ?	Anisimovienė, Merkys, 2000; Jodinskienė, 2005;
ABP kidney bean cytosol	7.8 7.8	– –	67 30	? ?	Mockevičiūtė, Anisimovienė, 1999;
ABP wheat cytosol	8.0	1.7×10^{-6}	40	Receptor	Даргинавичене, 1992
ABP wheat tonoplast	5.5 8.0	1.2×10^{-7} 1.8×10^{-7}	? ?	? ?	Merkys et al., 1988
ABP carrot plasmalemma	5.5	2×10^{-5}		Transporter	Zbell, 1996
AUX1	5.0-6.0	2.6 x 10^{-6}	?	Transporter	Carrier et al., 2008
ABP rice plasmalemma	5.5 5.5	4.5×10^{-7} 3.8×10^{-6}	? ?	Receptor Transporter	Zaina et al., 1989
Soluble mung bean ABP	7.0-7.6 5.0-6.0	2,4-D 9.3× 10^{-6} 2,4-D 9.5×10^{-6}	48 47, 15	Enzyme	Sakai, 1985
ABP maize membrane	7.0	1.0×10^{-6}	?	Enzyme	Nave, Benveniste, 1984
Soluble peach ABP 19/20	7.0	2,4-D 4.1×10^{-5}	19/20	Enzyme?	Ohmiya et al., 1993
Soluble peach Pp60	7.0	2,4-D 3.5×10^{-5}	60	Enzyme	Sugaya et al., 2000

The characteristics of specifically bound IAA-ABP complexes forming in chloroplasts and mitochondria organelles were more similar to complexes of soluble

proteins with enzymatic activities (Table 6), and also to ABPs identified in plasmalemma (Table 6), which were characterized as transporters.

However, it is difficult to consider about the role of ABPs functioning in those organelles basing only on the characteristics or similarity to other identified ABP complexes, the function of which are known or assumed. As it was already mentioned above, similar characteristic are of soluble ABP with enzymatic activities as well as of ABP – IAA-transporters functioning in plasmalemma, too. There is increasing evidence that plant tissues respond to a very high concentrations of auxin (Ohmiya et al., 1993). For example, the rate of transcription of auxin-regulated genes (*ARG1, ARG2*) of kidney bean hypocotyls increased as the concentration of IAA increased and reached a plateau at 10^{-4} M IAA (Yamamoto et al., 1992). The response of soybean *SAUR* to 2.4-D was not saturable up to 10^{-2} M (McClure, Guilfoyle, 1987). Basing on these data, Ohmiya et al. (1993) suggested that an auxin receptor(-ors) with a low affinity for auxin may exist in plant tissues.

All three ABP functioning in organelles as well as ABPs of kidney bean hypocotyls plasmalemma (Anisimovienė et al., 2000; Merkys et al., 2001; Jodinskienė, 2005; Jodinskienė, Anisimovienė, 2006) are proteins of low molecular mass. These data confirm the assumption that it is the sequence of amino acids in IAA binding to ABP site, but not a molecular mass of ABP predetermines the characteristics of forming complexes (Venis et al., 1992, Anisimovienė, Merkys, 2000; Christian et al., 2006). It was demonstrated that binding site – /-His-Arg-His-Ser-Cys-Glu-/- was characteristic of IAA receptor mediating in cell response to IAA by expanding (Venis et al., 1992; Jones, 1994; Anisimovienė, Merkys, 2000; Woo et al., 2001; Napier, 2004). The characteristics of forming IAA-ABP complexes were different when in IAA carboxylic group binding cluster between two amino acids /-His-/ instead of /-Arg-/ the other amino acid was localized (Ohmiya et al., 1998). Different characteristics were also established in TIR1-IAA complexes. This receptor having specific leucine-reach repeats for IAA and macromolecule interactions, is mediating transcriptional responses to auxin regulating plant development (Dharmasiri et al., 2005 b).

From the available results, it is difficult to estimate a possible role of IAA-ABP complexes forming in specific genetic information possessing cell organelles – chloroplasts and mitochondria. However, having in mind that IAA can be synthesized

and released into the culture medium as well as IAA metabolism (catabolism) processes may occur in their ancestors – bacteria's (Merkys et al., 1974; Sergeeva et al., 2002), and that these organelles retained their property to synthesize IAA (Fregeu, Wightman, 1983), whereas a chloroplast – to catabolize and translocate IAA (Sandberg et al., 1982; 1983; 1990; Brown et al., 1989; Sitbon et al., 1993; Anisimovienė et al., 2007), we presume that the role of chloroplast in the realisation of IAA hormonal function might be related to a physiologically active IAA form concentration regulation at cellular level. The consideration on a possible role of mitochondria in IAA hormonal system activity is most problematic, because this question has not been sufficiently investigated yet.

Further studies are needed to disclose the significance and possible functions of IAA-ABP complexes forming in cell organelles (chloroplasts and mitochondria) with respect to organelle and/or cell.

CONCLUSIONS

1. For the first time, the localization and functioning of auxin-binding proteins in kidney bean leaf chloroplasts and hypocotyls cells mitochondria was demonstrated at the organelles, their sub-compartments and proteins level.

2. Intactness and functionality of organelles were significant for IAA-ABP interaction peculiarities: functioning of different ABPs and formation of IAA-ABP complexes.

3. Two different specifically bound IAA-ABP complexes were forming in intact chloroplasts at medium pH optimum 5.5 and 7.5, and one complex – in intact mitochondria at medium pH optimum 7.0.

4. Localized in these organelles ABPs are distinguished in high auxin-binding activity, but low affinity for IAA ligand and possess a large number of binding sites. ABPs saturation with ligand reached plateau at high ^{14}C-IAA concentrations.

5. IAA and ABPs interaction in chloroplasts and mitochondria is biologically significant: physiologically active compounds competed for ^{14}C-IAA binding to ABPs sites, whereas ICA – an inactive IAA metabolite, did not show competitive activity.

6. Two different ABPs were functioning in intact chloroplast. One of these (native molecular mass was 50–53 kDa, complexes forming at pH optimum 5.5) was localized in stroma, another (native molecular mass was 38–42 kDa, complexes forming at pH optimum 7.5) – in membranes.

7. One protein functioning in mitochondria (native molecular mass was 45–47 kDa) was localized in membranes.

8. IAA-ABP complexes forming in chloroplasts and mitochondria were different according to their main characteristics (amount of IAA bound to protein unit, specificity of complexes, K_D, EC_{50}, n, ABP saturation with IAA, ligand specificity). They also differed from IAA-ABP complexes forming in kidney bean hypocotyls or wheat coleoptiles cells plasmalemma responding to IAA by elongation growth as well as from IAA complexes forming with ABP1 and TIR1.

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Indolil-3-acto rūgšties-baltymų kompleksai chloroplastuose ir mitochondrijose

Santrauka

Darbo tikslas: išaiškinti ASB buvimo ir funkcionavimo pupelių ląstelių organoiduose – chloroplastuose ir mitochondrijose galimybę.

Darbo uždaviniai:

- Išaiškinti IAR atpažįstančių ir specifiškai sąveikaujančių baltymų lokalizacijos ir funkcionavimo galimybę pupelių lapų ląstelių intaktiniuose chloroplastuose ir hipokotilių ląstelių zonos, atsakančios į IAR poveikį tįstamuoju augimu, intaktinėse mitochondrijose.
- Atskleisti IAR ir ASB sąveikos ypatybes chloroplastuose ir mitochondrijose. Charakterizuoti susiformuojančius kompleksus.
- Nustatyti, kuriame(-iuose) chloroplastų ir mitochondrijų subkompartmentuose gali būti lokalizuoti ASB.
- Charakterizuoti chloroplastų ir mitochondrijų subkompartmentuose susiformuojančius specifinius IAR-ASB kompleksus ir juos sudarančius ASB.
- Palyginti chloroplastuose ir mitochondrijose susiformuojančius IAR-ASB kompleksus su kituose ląstelių kompartmentuose susiformuojančiais IAR-ASB kompleksais.

IAR prijungimo saitų lokalizacijai ir IAR-ASB kompleksų formavimosi galimybių tyrimams buvo naudojamos >90 % švarumo ir ~97 % intaktiškumo pupelių hipokotilių mitochondrijų ir >90 % švarumo ir ~91,5 % intaktiškumo pupelių lapų chloroplastų subląstelinės frakcijos. Atlikus IAR ir ASB sąveikavimo galimybių tyrimus, pirmą kartą parodyta, kad intaktiškuose chloroplastuose funkcionuoja dvi IAR prisijungimo vietos – specifinė sąveika vyksta terpės pH esant 5,5 ir 7,5. IAR-ASB kompleksai kai terpės pH 7,5 formuojasi tik intaktiškuose ir funkcionaliuose chloroplastuose. Mitochondrijų intaktiniuose organoiduose aptikta viena IAR prisijungimo vieta, optimalus pH specifiškai sujungtų IAR-ASB kompleksų formavimuisi – 7,0. Nors mitochondrijų organoidų intaktiškumo ir funkcionalumo užtikrinimas neturi įtakos kitų IAR prisijungimo vietų funkcionavimui ir optimaliam IAR-ASB kompleksų formavimosi pH, tačiau turi įtakos ASB ir IAR sąveikai – bendrai ir specifiškai prijungtos ^{14}C-IAR kiekiui baltymo vienetui ir susiformuojančių kompleksų specifiškumui.

Specifinės IAR ir ASB sąveikos charakteristikų analizė parodė, kad organuoiduose susiformuojantys IAR-ASB kompleksai yra nevienodi. Visi organoiduose lokalizuoti ASB pasižymi dideliu specifiniu auksino prisijungimo aktyvumu, nors jų gimininguma IAR ligandui yra mažesnis nei daugelio žinomų ASB, kuriems priskiriama receptoriaus funkcija. Organoiduose lokalizuoti ASB turi didelį IAR prisijungimo vietų skaičių, o jų prisotinimas IAR ligandu, artėja prie pilno tik esant aukštoms ^{14}C-IAR koncentracijoms.

Tiriant ligandų konkuravimą dėl IAR prijungimo vietų nustatyta, kad pagal cheminę struktūrą IAR molekulei artimi indolo junginiai, t.y. junginiai turintys indolo žiedą ir karboksilo grupę trečioje padėtyje, dėl IAR prisijungimo prie chloroplastų ir mitochondrijų ASB konkuravo nevienoda tvarka: intaktinių chloroplastų preparatuose kai terpės pH 5,5: IAR>ISR>IPR>IPVR, o kai pH 7,5: IAR>ISR>IPVR>IPR. Intaktinių mitochondrijų preparatuose, terpės pH esant 7,0: IAR>IPVR>IPR>ISR. Kadangi dėl ^{14}C-IAR prijungimo vietų šiuose organoiduose konkuravo fiziologiškai aktyvūs junginiai, o

neaktyvūs IAR metabolitas IKR nekonkuravo, tai rodo, kad ši sąveika turi biologinę reikšmę. Antiauksinas ir auksino išnešimo iš ląstelės inhibitorius TJBR dėl ^{14}C-IAR prisijungimo vietų tiek prie abiejų intaktinio chloroplasto ASB, tiek prie intaktiniame mitochondrijų organoide funkcionuojančio ASB, konkuravo panašiai. Kitas IAR transporto inhibitorius – NFR ^{14}C-IAR iš prisijungimo prie ASB vietų aktyviai stūmė tik intaktinių chloroplastų frakcijoje, kai terpės pH 7,5. Intaktinių mitochondrijų organoiduose NFR konkuravo dvigubai silpniau, o chloroplastuose terpės pH esant 5,5 nekonkuravo visai.

Osmotinio šoko ir diferencinio centrifugavimo būdu atskyrus organoidų membranų struktūras ir skystų fazių (stromos bei matrikso) subkompartmentus nustatyta, kad IAR prisijungimo vieta chloroplastuose (optimalus kompleksų formavimuisi terpės pH 7,5) lokalizuota membranose. ASB, suformuojantis specifinius kompleksus su IAR kai terpės pH 5,5 lokalizuoti stromoje. Specifinių IAR atpažįstančių baltymų buvimo ir funkcionavimo galimybė mitochondrijų matrikse buvo atmesta, specifinė sąveika nevyko. Šiame organoide IAR-ASB kompleksų formavimasis, esant terpės pH 7,0, vyksta mitochondrijų membranų vezikulių preparatuose.

Įvertinus išsodintų amonio sulfatu tirpių chloroplastų stromos baltymų bei soliubilizuotų chloroplatų ir mitochondrijų membranų baltymų sąveikos su IAR charakteristikas nustatyta, kad visų šiuo atveju tirtų baltymų gimiringumas IAR ligandui yra didesnis, nei gimiringumas nustatytas intaktinių organoidų baltymų prie tokių pat pH reikšmių, o ^{14}C-IAR prisijungimo prie baltymo vietų skaičius mažesnis. Tiek išsodintų chloroplastų stromos, tiek soliubilizuotų chloroplastų membranų baltymų sąveikos su IAR K_D yra viena eile mažesnės, nei sąveikos su soliubilizuotais mitochondrijų membranų baltymais. Tai rodo, jog abiejų chloroplastų baltymų gimiringumas IAR ligandui yra didesnis nei soliubilizuotų mitochondrijų membranų ASB. Labai ženkliai tarp šių trijų ASB skiriasi ^{14}C-IAR prisijungimo prie baltymo vietų skaičius. Soliubilizuotų mitochondrijų ASB atveju, jis yra viena eile didesnis už IAR prisijungimo vietų skaičių prie išsodintų stromos ASB ir net dviem eilėm didesnis už IAR prisijungimo vietų skaičių prie chloroplasto membranose lokalizuotų ASB.

Artimos cheminės struktūros junginių gebėjimo išstumti ^{14}C-IAR iš kompleksų tiek su išsodintais chloroplastų stromos, tiek su soliubilizuotais membranų baltymais tyrimai parodė, kad abiem atvejais stūmimo aktyvumas ir eiliškumas atitiko intaktinių bei funkcionalių chloroplastų atvejais, prie tokių pat terpės pH, gaunamą stūmimo aktyvumą ir eiliškumą. IAR iš kompleksų, susiformuojančių su soliubilizuotais mitochondrijų membranų baltymais, skirtingai nei intaktiniame organoide, ISR išstūmė aktyviau, nei IPR.

Įvertinant organoiduose funkcionuojančių ASB molekulines mases nedenatūruojamosios PAAG elektroforezės metodu nustatyta, kad su IAR sąveikaujančių baltymų lokalizuotų chloroplastų stromoje molekulinė masė 50-53 kDa ribose, chloroplastų membranose funkcionuojančių ASB – nuo 38 iki 42 kDa. Mitochondrijų membranose aptiktų baltymų specifiškai atpažįstančių IAR molekulinė masė – 45-47 kDa. Nors abu chloroplastų (tirpus ir membraninis) ir vienas mitochondrijų (membraninis) ASB natyvioje būklėje yra mažos molekulinės masės baltymai, tačiau jų sąveikos su IAR charakteristikos (kompleksų specifiškumas, prijungtos IAR kiekis baltymo vienetui, K_D, EC_{50}, n, ASB prisotinimas IAR ir kt.) ženkliai skiriasi tarpusavyje. Šiuose organoiduose funkcionuojančių ASB charakteristikos ženkliai skiriasi ir nuo kitų pupelių tįstančių hipokotilių ląstelėse aptiktų ASB - dviejų plazmolemoje (Anisimovienė

et al., 2000; Merkys et al., 2004 a, b; Jodinskienė, 2005; Jodinskienė, Anisimovienė, 2006) ir dviejų citozolyje (Меркис и др., 1999; Mockevičiūtė, Anisimovienė, 1999; Anisimovienė et al., 2000) charakteristikų. Skiriasi ir nuo visuotinai pripažintų auksino receptorių sąveikos su auksinu charakteristikų – plazmolemoje funkcionuojančio ASB1 (Löbler, Klambt, 1985 a; Jones, 1994) ir tirpaus branduolio baltymo - TIR1 (Dharmasiri et al., 2005 a; Kepinski, Leyser, 2005) bei nuo kviečių koleoptilių ląstelių, atsakančių į IAR poveikį tįstamuoju augimu kompartmentuose – plazmolemoje, citozolyje, tonoplaste – susiformuojančių IAR-baltymų kompleksų (Merkys et al., 1988; Даргинавичене, 1992; Anisimovienė et al., 1998; Darginavičienė, Novickienė, 2002). Šie duomenys leidžia daryti prielaidą, kad šiuose organuoduose susiformuojančių IAR-ASB funkcijos gali būti kitokios nei IAR-receptorinių kompleksų plazmolemoje ar branduolyje. Tam, kad būtų atskleista IAR-ASB kompleksų, susiformuojančių chloroplastuose ir mitochondrijose, reikšmė ir galimos funkcijos – organoido ir/ar ląstelės atžvilgiu reikalingi tolimesni tyrimai.

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