

Analysis of compartmentation of auxin binding proteins in kidney bean cell mitochondria

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The localization of auxin binding sites in mitochondria sub-compartments (matrix – liquid phase and membranous structures) fractions separately has been investigated for the first time. The specimens of matrix and membranous structure fraction have been prepared by osmotic shock and differential centrifugation procedures.

The analysis of IAA-ABP (indole-3-acetic acid-auxin binding protein) complexes formation in these fractions as well as protein samples derived from matrix by precipitation with ammonium sulphate and purified on column chromatography (gel-filtration) or solubilized membranous protein specimens were performed at the circumstance used for IAA-ABP complexes formation in intact mitochondria.

On the basis of the obtained experimental results the preliminary supposition that the possibility of ABPs functioning in matrix is doubtful may be suggested. According to preliminary results, the IAA proteins are possibly localized in membranous structures and auxin binding activity may be attributed to integral membranous proteins. The possibility of formation of specifically bound IAA-ABP complexes with solubilized by digitonin membranous proteins at pH 7.0 having the same or analogous specificity as the IAA-ABP complexes formed in intact mitochondria has been demonstrated.

Key words: indole-3-acetic acid (IAA), IAA binding sites, IAA-ABP complexes, mitochondria, kidney bean.

Introduction. According to the current endosymbiotic theory, plant cell organelle mitochondria as well chloroplast are of endosymbiotic origin (Gray et al., 1999). Chloroplast comes from photosynthetic green algae – free-living cyanobacteria (McFadden, 2001), while the mitochondria – from α -proteobacteria, rickettsias (Gray et al., 1999).

These organelles have their own genetic apparatus – not identical to nucleus, which during evolution path has been reduced and (or) modified (Gray et al., 1999). Some bacterial genes from mitochondria ancestors, such as central intermediary metabolism, cell envelope formation, biosynthesis of cofactors and others, were lost or transferred to the nucleus (Martin, Herrmann, 1998; Race et al., 1999) or (and) replenished by introducing chloroplast DNA sequences (Lacoste-Royal, Gibbs, 1985). Thus mitochondrial genome retained the possibility to respiration, transcription and

translation of few own proteins (about 8–16), mostly related with respiratory function (Igmaberdiev, 2000).

Besides, employing physical-chemical methods of phytohormones analysis and identification, it was determined that bacteria have the possibility to synthesize, in contrast to flowering plants, high amount of phytohormone indole-3-acetic acid (IAA) and to separate it out of the cell into culturall medium (Libbert et al., 1969; Merkys et al., 1974). At present this feature is confirmed employing modern methods of phytohormones analysis, such as HPLC, mass spectrometry (Sergeeva et al., 2002; Karadeniz et al., 2006).

Nevertheless, there are still no suggestions on the possible role of IAA in this organelle. So far the presence of endogenous IAA was discovered in mitochondria sub-cellular fraction derived from sunflower leaves by method of gas chromatographic analysis (Fregeau, Wightman, 1983). According to the data of these investigators the level of endogenous IAA found in mitochondria is lower in comparison to chloroplast. The possibility of IAA localization in mitochondria has been also confirmed employing immunocytochemical method (Ohmiya, Hayashi, 1992). There is only one publication with experimental evidence on the possibility of IAA synthesis in sub-cellular mitochondria fraction derived from sunflower leaves from the precursor amino acid L-tryptophan (Fregeau, Wightman, 1983).

Based on available information that: a) mitochondria is of bacterial origin; b) the IAA synthesis feature is characteristic for both bacteria and mitochondria; c) this organelle has its own genetic apparatus and can synthesize several individual proteins, the investigations of the localization of auxin binding proteins (ABPs) in mitochondria have been started. The obtained results revealed that one IAA binding site of is functioning in intact mitochondria (Anisimovienè et al., 2004). Optimal pH for IAA-ABP complexes formation is 7.0.

The aim of the present investigations here is to determine, in which compartment of mitochondria matrix or membranes the ABP are functioning.

Object, methods and conditions. Etiolated 4–5-day age kidney bean (*Phaseolus vulgaris* L.) hypocotyl segments responding to IAA influence by elongation growth were used as the test object for the investigation of ABPs localization in plant cells mitochondria sub-cellular fraction.

The optimal conditions for intact and functional mitochondria sub-cellular fraction isolation: homogenization and wash medium compositions, differential centrifugation, purification on a two-step Percoll gradient were carried out according to procedures described by Jackson et al. (1979), and Fregeau and Wightman (1983) and adjusted to test object (Anisimovienè et al., 2004) in order to prepare sub-cellular fraction of mitochondria, which ensured purity and intactness.

The intactness and functionality of the mitochondria sub-cellular fraction was estimated by measuring the concentration of oxygen using a chamber with a Clark-type electrode (Jackson et al., 1979). The possible contamination of mitochondria fractions the other compartments of the cell was verified biochemically. Protein content was determined by Bradford (1976) method.

For sub-fractionation of intact mitochondria specimens the osmotic shock procedures by three freeze (at -70 °C) and thaw (at 4 °C) in hypotonic medium

10 mM TRIS-HCl, pH 8.0, 10 mM TRIS-HCl, pH 7.0, 1 mM EDTA and 1 mM PMSF (ratio – 1 : 7) cycles succeeding have been used (Fig. 1).

Separation of both membrane and matrix fractions was achieved by two different procedures. The membranes and matrix sub-fractions were differentiated from each other by centrifugation for 10 min at 16 000 g (Möller et al, 1993). Or total mitochondria samples (containing membranes and matrix mixture) obtained after freezing and thawing procedure were vigorously shaken for 5 min with refined sunflower oil, then separated into mitochondrial membrane structures containing their own proteins, aqueous phase enriched with mitochondrial matrix proteins and protein free oil phases by centrifugation at 20 000 g × 10 min (Hájek et al., 2004).

In order to reveal, in which sub-compartment of mitochondria the phytohormone IAA binding sites are localized, the prepared membrane and matrix sub-fractions were used for investigation of IAA with ABPs interaction (Fig. 1).

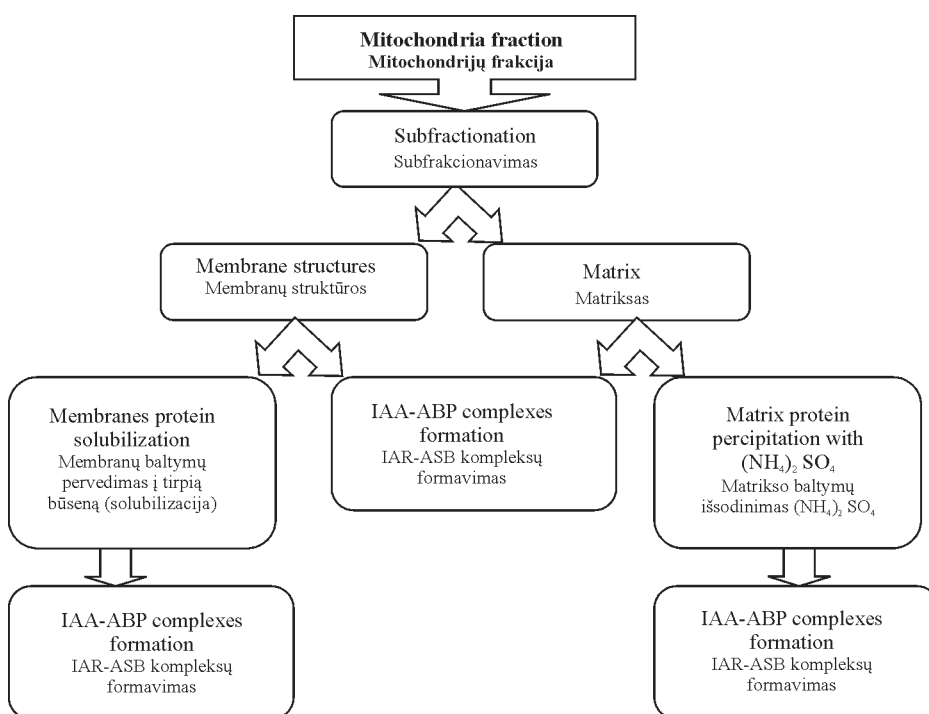


Fig. 1. The principal scheme of IAA binding activity in mitochondria sub-compartments analysis

1 pav. Principinė IAR sujungimo aktyvumo mitochondrijų subkompartmentuose tyrimų schema

IAA binding assays were carried out by common methods used for IAA and ABP interaction analysis with other compartments of the cell, namely cytosol, plasmalemma, chloroplast, tonoplast (Merkys et al., 1998; Anismovienė et al., 2000).

In such cases identical binding medium as for IAA-ABPs interaction analysis in intact mitochondria has been used (Anisimovienė et al., 2004). It consists of 50 mM TRIS-HCl buffer pH 7.0, containing 1 mM KCl, 1 mM MgCl₂. Exposition time – 30 min. For assessment of total incorporation of exogenous IAA into complexes the (2 – ¹⁴C) 3-indoleacetic acid (American Radiolabeled Chemicals, Inc. 384) at concentration of 0.5 μM (0.0005 mM) was used and specificity of forming complexes was determined by using no-labeled IAA concentration of 0.1 mM. The formed IAA-ABP complexes were precipitated with trichloroacetic acid or by filtration through membrane filters (0.2 μm, Millipore) (Fig. 1).

The amount of total and specifically bound IAA in complexes was estimated by measuring the radioactivity of ¹⁴C-IAA cpm (counts per minute) in Bray's system with scintillation counter Beckman LS 1801. K_d – by the calculation of unlabelled IAA concentration needed for 50 % displacement of specifically bound ¹⁴C IAA (Ray et al., 1977).

Results. Analyzing the possibility of ABP localization and functioning in kidney bean mitochondria sub-cellular fraction, it was revealed that in intact mitochondria IAA-ABP complexes are formed at pH optimum 7.0. Specificity of these complexes is 41.32 ± 3.62 %, amount of ¹⁴C-IAA binding with protein unit 8 346 ± 435 cpm × mg⁻¹ protein. Amount of total and specifically bound IAA with protein unit about 2 times higher than total and specificity amount of intact chloroplast at pH 7.5, but complexes specificity is similar (Anisimovienė et al., 2004; Merkys et al., 2004; Mockevičiūtė et al., 2006). Comparison of these and other main characteristics of IAA-ABP complexes formed in different compartments of cells: KD, number of IAA binding sites – n, saturation of IAA binding sites (Merkys, Darginavičienė, 1997; Anisimovienė et al., 2000) revealed that IAA-ABP complexes formed in intact mitochondria are different from IAA-ABP complexes formed in other plant cell compartments.

In order to estimate the locality of IAA binding sites in mitochondria the derived intact specimens of these organelles were divided into two primary (basic) sub-compartments by osmotic shock procedure. Matrix – liquid phase – of organelle, in which synthesized polypeptides, encoded by mitochondrial DNA (Smeitink, 2003), and membranous fractions where the translocase machinery of proteins import (Zhang, Glaser, 2002) and metabolite transporters – import/export (Picault, 2004) are localized and functioning.

As mentioned above, both isolated mitochondria sub-cellular fractions for IAA binding tests were applied separately according to procedures commonly used for IAA-ABP interaction analysis in other compartments of the cell, including intact mitochondria (Merkys et al., 2004; Anisimovienė et al., 2004), i. e. in optimal for IAA-ABP complexes formation binding medium and pH 7.0, amount of protein 300 μg and 0.5 μM IAA in each sample of matrix or membranes.

According to the obtained results (Fig. 2), amount of IAA specifically associated with ABP unit (cpm × mg⁻¹ protein) in matrix is very low and reaches only ~ 5 % in comparison with amount of specifically bound IAA to such amount of protein in intact mitochondria. Specificity of these complexes is faithless, too. It reaches only about 2–3 %.

In order to ascertain if the destruction of organelle has no effect on the value of optimal pH for IAA and ABPs interaction, the analysis of IAA-ABP complexes formation possibility in matrix was carried out at different pH from – 4 to 8 every 0.5. In such cases the reliability of specific IAA recognizing proteins residence and functioning in mitochondria matrix as well as the possibility of specifically bound IAA-ABP complexes formation in this sub-compartment of tested organelle has not been supported. The amount of IAA bound with 1 mg of ABP ranges from 1 120 to 2 080 cpm specificity of formed complexes only from 1.8 to 3.5 %.

Tenuous possibility of specific IAA-ABP interaction and auxin recognizing protein functioning in matrix was confirmed when the IAA binding experiments were performed employing specimens of this mitochondria sub-compartment proteins precipitated by ammonium sulphate (up to 80 % saturation) and purified by gel-filtration on Sephadex G-25 column. Specific binding activity of purified protein specimens reaches about 6–7 % in comparison with intact mitochondria protein unit.

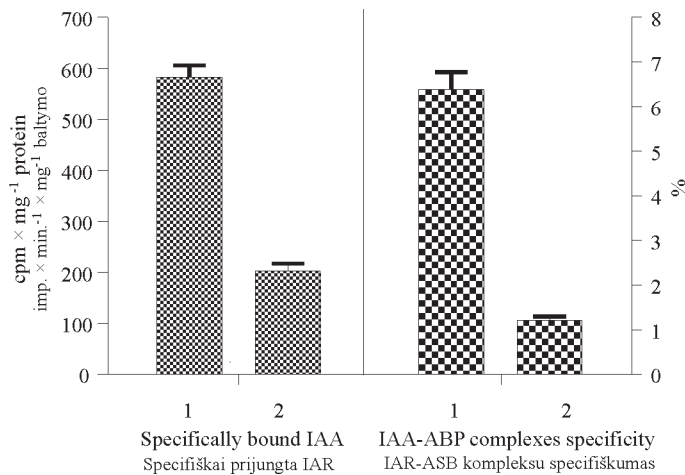


Fig. 2. Comparison of IAA binding activity in mitochondria sub-fractions
2 pav. IAR sujungimo aktyvumo mitochondrijų subfrakcijose palyginimas

Thus, the obtained results led us to supposition that the possibility of ABP functioning in matrix is doubtful.

Data of analysis of IAA-ABP complexes formation with crude membranous structures specimens reveals the possibility of auxin binding sites localization in this sub-compartment of mitochondria (Fig. 2). Although IAA and ABPs interactions are weak the amount of IAA bound with protein unit is higher about 2–3 times in comparison with matrix and reaches $4\ 162 \pm 220$ cpm × mg⁻¹ protein. The value of specificity of formed complexes in membranes exceeds 7–10 %. The results of supplementary experiments on the effect of different pH (from 4 to 8, every 0.5) confirmed that the specifically bound IAA-ABP complexes formation in mitochondria membranes

specimens is subordinated to pH 7.0. These results allow a supposition that proteins of mitochondria membrane may be involved into formation of IAA-ABP complexes in this organelle. This feature was confirmed by results of the experiments when other methodical approaches have been used for mitochondria sub-fractionation (Hájek et al., 2004). In this case sub-fractionation of mitochondria into membranes and matrix fractions was achieved by their distribution between polar and non-polar phases employing sunflower oil. It is known that under this procedure the membrane structures are not destroyed (Hájek et al., 2004). In such experiments the amount of IAA bound with membrane specimens reaches $5\,716 \pm 390$ cpm and complexes specificity 12 %, i. e. it is higher.

Based on these results the following supposition has been formulated: the mitochondria's ABPs are localized and functioning in membranes. Therefore, to deny or confirm this presumption, the experiments employing solubilized membrane protein specimens are preferable (Jones, 1999; Anisimovienė, Merkys, 2000; Jodinskienė, Anisimovienė, 2007). At initial stages of these experiments the non-ionic detergent Triton X-100, convenient for plasmalemma integral proteins solubilization has been chosen. The optimal concentration of detergent (from 0.5 to 2.0 %) as well as the protein and detergent ratio (from 1 : 1 to 1 : 5) have been selected experimentally by comparison of the main characteristics of IAA-ABP complexes formed in intact mitochondria (Table).

Table. Comparison of IAA-ABP complexes characteristics
Lentelė. IAR-ASB kompleksų charakteristikų palyginimas

Test object Testo objektas	Amount of specifically bound ¹⁴ C-IAA, cpm × mg ⁻¹ protein Specifinis ¹⁴ C-IAR prisijungimas imp. × min. ⁻¹ × mg ⁻¹ baltymo	IAA-ABP complexes specificity IAR-ASB kompleksų specifiškumas (%)
Intact mitochondria Sveikos (funkcionuojančios) mitochondrijos	8 346 ± 435	38–41
Mitochondria membranes specimens Mitochondrijų membranų preparatai	5 716 ± 390	12
Mitochondria membranes protein solubilized with Triton X-100* Mitochondrijų membranų baltymai, solubilizuoti Tritonu X-100*	6 254 ± 361	14–15.5
Mitochondria membranes protein solubilized with Digitonin** Mitochondrijų membranų baltymai, solubilizuoti Digitoninu**	7 499 ± 482	30–40

* Concentration of Triton X-100 1.75 %, detergent and protein ratio 1 : 3
Tritono X-100 koncentracija – 1,75 %, detergento baltymo santykis 1 : 3

**Concentration of digitonin 1.0 %, detergent and protein ratio 1 : 5
Digitonino koncentracija – 1,0 %, detergento baltymo santykis 1 : 5

Although under high detergent Triton X-100 concentration and high protein : detergent ratio implication we did not succeed to achieve the expected results. Apparently, we did not achieve full solubilization of these membrane integral proteins (Jones, 1999; Anisimovienė, et al., 2000). Because, when for solubilization of these proteins detergent digitonin (Eubel et al., 2003) 1 % concentration, specific for mitochondria membranes solubilization was applied, the same or almost the same amount of IAA specifically bound with ABP unit and specificity of formed complexes have been determined (Table).

Discussion. During the last two decades auxin binding sites localization on plasmalemma and cytosol, IAA-protein interaction peculiarities as well as the role of these complexes in auxin hormonal system action have been widely investigated (Darginavičienė, 1992; Merkys et al., 1998; Anisimovienė et al., 2004; Zazimalova, Napier, 2003).

Earlier experiments (Merkys et al., 2003; Anisimovienė et al., 2004; Mockevičiūtė et al., 2006; Jodinskienė, Anisimovienė, 2007) revealed at least seven different binding sites functioning in dicot (kidney bean) plant cells. Two of them are functioning in plasmalemma (optimal pH for IAA-ABP complexes formation 5.5 and 7.5). Two IAA-ABPs complexes are formed cytosol. The optimal pH for both low affinity (molecular mass > 67 kDa) and high affinity (\approx 30 kDa) binding sites is 7.8. Two IAA-ABP complexes (optimal pH 5.5 and 7.5) formed in kidney bean leaves cells chloroplast were also ascertained (Anisimovienė et al., 2004). Based on experimental data the presumption that IAA binding sites, which formed IAA-ABP complexes at pH optimal 5.5 are localized and functioning in stroma, while other formed in chloroplast (optimum pH for IAA-ABP complexes formation at 7.5) may be related to the proteins localized in membranous structures (Mockevičiūtė et al., 2006) has been formulated. One binding site is localized in mitochondria (Anisimovienė et al., 2004).

Presented results support the possibility of ABPs functioning in membranes of this organelle. These results may be considered as to be in agreement with the date obtained by other investigators, in which the possibility of IAA synthesis in mitochondria from amino acid tryptophane has been determined. The occurrence of natural IAA in both plant cell organelles – chloroplast and mitochondria – in sunflower, barley leaves using methods of biochemical analysis and in leaf cells of peach using immunocitochemical analysis (Wighman, Fregeau, 1983; Ohmyia, Hyashi, 1992) has been proved. They may be also be considered in agreement with data on the possibility of bacteria – according to endosymbiotic theory progenitors of these organelles in plant cell (Gay, 1999) – to synthesize high amounts of IAA and segregate this compound out of cell (Libbert et al., 1969; Merkys et al., 1974; Sergeeva et al., 2002; Karadeniz et al., 2006).

In spite of the discovery of the possible of IAA-ABP complexes formation in intact and functioning mitochondria (Merkys et al., 2004; Anisimovienė et al., 2004) and localization of IAA binding sites in their membranes of it, the consideration on the possible role of these complexes formed in this organelle in IAA system action or signaling is problematic because these questions are still no investigated.

While the characteristics of IAA-ABP complexes formed in mitochondria of kidney bean cells differ from ABP-IAA-receptor complex, having the carboxyl group binding domain /-His-Arg-His-/ and functioning in plasmalemma, it is possible to

assume that their role also differs. Characteristics of IAA-ABP complexes formed in mitochondria membranes are comparable with specifically bound IAA complexes formed with other cupin family ABP, named ABP19/20, localization of which in mitochondria of peach leaves cells has been determined immunocytochemically (Ohmyia et al., 1998). In this ABP IAA carboxyl group binding cluster between two histidines the redundancy of amino acid threonine is localized. Doubtless, the IAA signaling and redistribution in the cell involves many proteins, not just IAA-receptors (Teale et al., 2006). Realization of IAA physiological function in growth and developmental processes of the plant cells is related not only with interaction of ABP- proteins receptors but also with other IAA-recognizing proteins, namely IAA-enzymes and IAA-transporters (Zazimalova, Napier, 2003; Teale et al., 2006). Based on the possibility of bacteria cell translocation of IAA out of the cell (Merkys et al., 1974; Sergeeva et al., 2002; Karadeniz et al., 2006), it may be supposed that IAA could be portaged out of mitochondria into cytosol by amino acids permeases such as ANT1, member of LAX family transporters having 11 putative transmembrane domains (Chen et al., 2001; Teale et al., 2006). This feature may be related to structural similarity of IAA with its precursor amino acid tryptophan and histidine (Zazimalova, Napier, 2003). IAA transport through organelle (chloroplast and mitochondria) membranes may be performed by bacterial ancestor permeases – amino acid/auxin permeases characteristic for plants, animals, yeast and fungi cells (Young et al., 1999) as well.

In spite of that the question on the possible role of IAA-ABP complexes formed in mitochondria membranes must to be answered in future.

Conclusions. 1. Our results for the first time show that ABP formed complexes with IAA in intact mitochondria at optimal pH 7.0 may be localized in membrane structures of this organelle.

2. Because the same or analogous IAA binding activity of ABP in intact mitochondria has been achieved in specimens of membranes proteins fully solubilized by digitonin, it is possible to assume that this feature is characteristic for integral membrane proteins.

3. The possibility of ABPs localization and functioning in matrix remains doubtful.

Acknowledgements. This work was in part supported by Lithuanian State Science and Studies Foundation.

Gauta 2009 07 15

Parengta spausdinti 2009 12 07

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SODININKYSTĖ IR DARŽININKYSTĖ. MOKSLO DARBAI. 2009. 28(4).

Auksiną prisijungiančių baltymų kompartmentacijos pupelių ląstelių mitochondrijose tyrimai

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Santrauka

Pirmą kartą tirta auksino prisijungimo vietų lokalizacija mitochondrijų sub-kompartmentų (matrikso – skysta fazė ir membranų struktūrų) frakcijose atskirai. Matrikso ir membranų struktūrų frakcijų preparatai buvo gauti osmotinio šoko ir diferencinio centrifugavimo būdu.

Šiose frakcijose ir matrikso baltymų, išsodintų amonio sulfatu ir išvalytų chromatografinėje (gel-filtracinėje) kolonėlėje, ar solubilizuotų membranų baltymų pavyzdžiuose IAR-ASB (indolil-3-acto rūgštis – auksiną prisijungiantys baltymai) kompleksų formavimosi analizė buvo atlikta tomis pačiomis sąlygomis, kaip ir tiriant IAR-ASB kompleksų formavimąsi intaktinėse mitochondrijose.

Sukauptų bandymų rezultatų duomenys leido daryti prielaidą, kad ASB funkcionavimo galimybė matrikse yra abejotina ir galbūt gali būti atmesta.

IAR atpažįstantys baltymai, matyt, yra lokalizuoti membranų struktūrose, o auksino prisijungimo aktyvumas priskirtinas integraliniams membranų baltymams.

Parodyta specifiskai sujungtų IAR-ASB kompleksų formavimosi galimybė digitoninu solubilizuotais membranų baltymais, kai terpės pH 7,0. Susiformavę IAR ir mitochondrijų membranų baltymų kompleksai yra tokio pat arba analogiško specifiskumo, kaip ir IAR-ASB kompleksai, susiformuojantys intaktinėse mitochondrijose.

Reikšminiai žodžiai: indolil-3-acto rūgštis (IAR), IAR prisijungimo vietos, saitai, IAR-ASB kompleksai, mitochondrijos, pupelės.