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NOVEL INTERACTIONS OF ADRENODOXIN-RELATED [2Fe-2S] PLANT FERREDOXINS MFDX1 AND MFDX2 INDICATE THEIR INVOLVEMENT IN A WIDE SPECTRUM OF FUNCTIONS IN PLANT MITOCHONDRIA

Elena K. Shematorova[1], Ivan Yu. Slovokhotov[1], Vladimir N. Shmakov[2], Marat R. Khaliluev[3], Dmitry G. Shpakovski[1], Valery N. Klykov[1], Olga G. Babak[4], Svetlana G. Spivak[4], Yuri M. Konstantinov[2,5], and George V. Shpakovski[1,#]

[1] Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Moscow, RUSSIA

[2] Siberian Institute of Plant Physiology and Biochemistry SB RAS, Irkutsk, RUSSIA

[3] All-Russian Scientific Institute of Agricultural Biotechnology, Moscow, RUSSIA

[4] Institute of Genetics and Cytology, National Academy of Sciences of Belarus, Minsk, BELARUS

[5] Irkutsk State University, Irkutsk, RUSSIA

[#] Corresponding author, gvs@ibch.ru

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*Electron transfer chains of plant organelles (both chloroplasts and mitochondria) contain their own special set of ferredoxins. The relatively recently described adrenodoxin-like [2Fe-2S]-ferredoxins MFDX1 and MFDX2 of plant mitochondria are among the least studied of these. Until now, the only established function for them is participation in the final stage of biotin biosynthesis. In this work, using genetic and biochemical approaches, we searched for possible partners of these proteins in the genomes and proteomes of tobacco (*Nicotiana tabacum *L.) and foxglove (*Digitalis purpurea *L.) plants. MORF9 protein, one of the auxiliary components of the RNA editing complex of organelles (editosome), was found among the most prominent protein partners of adrenodoxin-like [2Fe-2S] tobacco ferredoxins. According to the results obtained from the yeast two-hybrid system, NtMFDX1 and NtMFDX2 of tobacco also bind and interact productively with the previously uncharacterised long non-coding polyadenylated RNA, which, based on its structural features, is capable of regulating the function of a number of components of complexes I (Nad1, Nad5) and III (protein of the cytochrome c synthesis system CcmF) and contributes to the formation of Fe/S-clusters in the corresponding protein complexes of the respiratory chain of plant mitochondria. We found one of the main components of the thiazol synthase complex (mitochondrial protein DpTHI1) to be the partner of ferredoxin DpMFDX2 of* Digitalis purpurea*. Finally, additional arguments were obtained in favour of the possible participation of MFDX1 and MFDX2 in the very ancient, but only recently described 'progesterone' steroid hormonal regulatory system: in leaves of the previously constructed CYP11A1-transgenic tomato plants, only the mature form of mitochondrial cytochrome P450scc (CYP11A1) of mammals is able to enter the mitochondria, where the above-mentioned components of the electron transport chain are localised. In summary, all of the newly revealed interactions of adrenodoxin-like [2Fe-2S] ferredoxins MFDX1 and MFDX2 indicate their participation in a wide range of functions in plant mitochondria.*

Key words: *CYP11A1, cytochrome P450scc, mitochondrial ferredoxins MFDX1 and MFDX2, transgenic plants.*

INTRODUCTION

Ferredoxins are a large family of small hydrophilic iron-sulphur-containing proteins belonging to the class of oxidoreductases. The catalytic centre is represented by an iron-containing cluster capable of taking one electron from FAD-containing ferredoxin reductase and donating it to acceptors of various types. Ferredoxins are divided into sev-

eral subfamilies depending on the type of cluster and homology of amino acid sequences. Ferredoxins with a molecular weight of 6-25 kDa and as a rule containing a [2Fe-2S]-type cluster were found in cytochrome P450-dependent monooxygenase systems. Plant ferredoxins (FDX) containing iron-sulphur clusters 2Fe-2S, can be divided into two classes according to their localisation: plastid and mitochondrial (Takubo *et al*., 2003; Ohta and Mizutani, 2004). For example, in the model plant *Arabidopsis thaliana*, of the nine genes encoding [2Fe-2S]-FDXs, six encode chloroplasts' FDXs, and three — mitochondrial type ferredoxins (MFDXs), the primary structures of which in two show clear similarity with the adrenodoxins of mammals (bovine, man). The real functions of adrenodoxin related mitochondrial ferredoxins of plants have still almost not been studied. At present, the largest database of protein-protein interactions (BIOGRID) for these proteins does not provide such interactions, even for the model plant *Arabidopsis thaliana*.

The aim of the present work was to study the interactome and identify new (and, if possible, the whole spectrum of) functions *in vivo* of two recently described mitochondrial ferredoxins (MFDX1 and MFDX2) of tobacco plants (Shematorova *et al*., 2014) and MFDX2 of *Digitalis purpurea*, which has obvious structural similarities with mammalian adrenodoxin and appears to be involved in the recently discovered progesterone system of hormonal regulation in higher plants (Shpakovski *et al*., 2017). Specifically for this purpose we constructed a series of vectors and representative cDNA libraries for the respective plant species, which are compatible with the yeast two-hybrid Interaction Trap system (Golemis *et al*., 2009; Proshkin *et al*., 2011, Shematorova *et al*., 2013). The most significant of the detected interactions were confirmed by independent biochemical methods. The identified interactions indicate important new functions of adrenodoxin related [2Fe-2S] ferredoxins MFDX1 and MFDX2 in redox processes involving electron transfer chains of plant mitochondria. Having proved mitochondrial localisation of mature cytochrome CYP11A1 in transgenic tomato plants and its ability at least transiently to interact with MFDXs, we also demonstrated the principal possibility of existence and successful functioning of 'mitochondrial' P450 cytochromes in plants.

MATERIALS AND METHODS

Plant material. The homozygous *CYP11A1* transgenic tobacco line TR-7, used in this study for YTH-system compartible cDNA library construction, was characterised previously (Kartel *et al*., 2004; 2007; Spivak *et al.*, 2009). The tobacco seeds were surface sterilised in 96% ethanol for 10 s and in 20% solution (v/v) of the commercial bleach 'Ace' with a few drops of Tween-20 for 15 min, then rinsed with sterilised distilled water six times for 1 min each. After surface sterilisation, the seeds were cultured on the Murashige and Skoog (MS) basal medium (Murashige and Skoog, 1962) without growth regulators supplemented with 3% (w/v) sucrose and 0.8% (w/v) agar. The pH was adjusted to 5.8 before autoclaving. The cultures were maintained under 23 ± 1 °C, with fluorescence light (65 μmol m^{-2} s^{-1}) during the long-day photoperiod (16 h light/8 h dark).

Transgenic lines of tomato (*Solanum lycopersicum* L., syn. *Lycopersicon esculentum* Mill.) carrying *CYP11A1* cDNA expressing the P450scc (CYP11A1) gene (lines 1, 2, 3, 4, 5, 7) were described in Shpakovski *et al*., 2017. Cell cultures from these lines and the wild type of tomato (cultivar Recordsmen) were prepared from friable calli (see below) and cultivated on Murashige and Skoog medium with the addition of 3% (w/v) sucrose, 0.7% (w/v) agar, 2.0. mg/l dichlorophenoxyacetic acid (2.4.D), 0.2 mg/l benzyladenine (BA). In the case of tomato transgenic lines the cultivation medium contained also 25 mg/l kanamycin. Cultivation was carried out in the dark at a temperature of 24 °C. The subculturing period was 28 days.

Formation of friable callus culture in tomato. Tomato leaves of wild-type (WT) seedlings (*Solanum lycopersicum* L. cv. Rekordsmen) and from six putative transgenic lines (lines 1, 2, 3, 4, 5, 7) expressing full sized *CYP11A1* cDNA of cytochrome P450scc from the bovine adrenal cortex were used for callus induction. For callus induction, leaves of transgenic tomato plants were cultured on agar-solidified MS selection medium containing 2 mg/l zeatin and 0.1 mg/l indole-3-acetic acid (IAA), 25 mg/l kanamycin and 150 mg/l timentin. Leaves of wild-type (WT) tomato plants were cultured on a medium of the same concentrations of plant growth regulators without the addition of a selective agent. The forming compact globular callus of light green colour was subcultured in 300 cm^3 glass culture vessels containing agar-solidified MS medium supplemented with 2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.5 mg/l 6-benzylaminopurine (6-BA) without kanamycin. Friable callus tissue was transferred to fresh culture medium every 15 days for a period of two months.

Isolation of plant mitochondria from tomato cell culture. Calli lines of tomato (*Solanum lycopersicum* L., syn. *Lycopersicon esculentum* Mill.) from wild type and transgenic lines, carrying *CYP11A1* cDNA expressing the P450scc (CYP11A1) gene (lines 1, 2, 3, 4, 5, 7), were cultivated on Murashige and Skoog medium with the addition of 3% (w/v) sucrose, 0.7% (w/v) agar, 2.0 mg/l dichlorophenoxyacetic acid (2.4.D), 0.2 mg/l benzyladenine (BA). In the case of tomato transgenic lines the cultivation medium contained also 25 mg/l kanamycin. Cultivation was carried out in the dark at a temperature of 24 °C. The subculturing period was 28 days. Mitochondria were isolated from tomato (*Solanum lycopersicum*) callus tissue cells according to Brown and Thorpe (1982) with some minor modifications. Callus cell culture at the age of 28 days was used for the mitochondria isolation. The extraction buffer for this procedure contained 400 mM sucrose, 2 mM EDTA, 0.5% (w/v) BSA, 0.6% (w/v) polyvinylpyrrolidone 40000, 0.1% β-mercaptoethanol, 20 mM cysteine, 20 mM KH_2PO_4 (pH 7.4).

Mitochondria solubilisation before subsequent Western bloting procedure. The suspension of mitochondria was thawed after storage and one volume of solubilisation buffer of the following composition was added: 62.5 mm Tris-Hcl, pH 6.8, 1 mm EDTA, 1% dodecyl sulfate Na, 20% glycerin (v/v), 10% β-mercaptoethanol (v/v). After adding solubilisation buffer, the sample was placed in a water bath at 97 °C for 5 min and then centrifuged at 10 000 g for 15 min.

Construction of vectors and plant (*Nicotiana tabacum* L. and *Digitalis purpurea* L.) cDNAs libraries for YTH systems. The initial vector for the YTH Interaction Trap system pJG4-5 (6449 bp) was modified using oligonucleotide adaptors with different SfiI sites that allow directional cloning of cDNA fragments in all three reading frames. As a result, plasmid vectors pGAA-SfiI (6476 bp), pAAT-SfiI (6490 bp) and pATT-SfiI (6486 bp) were obtained, a mixture of equal aliquots of which were used to produce cDNA libraries. The total RNA was extracted from the leaves of plants frozen and homogenised in liquid nitrogen using the TRIzol method (Invitrogen) and purified using the Qiagen RNeasy MinElute Cleanup kit (Qiagen). Full-size cDNAs were synthesised according to the SMART method (Zhu *et al*., 2001), using a system of nested SMART primers with different SfiI sites (at 5'-and 3'-ends of cDNAs) and Long Distance PCR amplification (Barnes, 1994) for synthesis of the second chain and production of double-chain cDNAs. The cDNA expression libraries are able to produce in yeast fragments of various proteins from the tobacco and digitalis plants as polypeptides fused with the synthetic acidic activation domain B42 ("acid blob"; 88 aa in length) (Ruden *et al*., 1991). Estimated working parameters of the obtained cDNA libraries are the following: 2.6×10^6 independent clones with average cDNA insert size 1 kb for *Nicotiana tabacum* library and 2.1×10^6 independent clones, average cDNA insert size 0.7 kb for *Digitalis purpurea*.

Yeast two-hybrid screening. Interactions of examined plant mitochondrial ferredoxins with various proteins of tobacco or foxglove proteome *in vivo* were studied with the use of a current version of the yeast two-hybrid system Interaction Trap (Gyuris *et al*., 1993; Serebriiskii *et al*., 2001; Golemis *et al*., 2009) with some minor modifications described in Shematorova *et al*., 2013.

Heterologous expression of cDNAs encoding adrenodoxin related [2Fe-2S] plant ferredoxins MFDX1 and MFDX2 in the bacterial system and other molecular biology techniques. For heterologous expression of cDNAs encoding adrenodoxin related [2Fe-2S] plant ferredoxins MFDX1 and MFDX2 in the bacterial system and to confirm protein–protein interactions detected by a two hybrid system *in vitro*, we used the vector system (pEXPR1 previously developed in our laboratory in combination with different pET plasmids from Qiagen, Germany) of compatible replicons supporting two plasmids in a single bacterial cell and allowing effective coexpression of various cDNAs presumably encoding the interacting proteins (Proshkin *et al*., 2011). The vector system we developed allows also to induce synthesis of a single or even both proteins of interest in *E. coli* and their rapid purification by metal-affinity chromatography on Ni-agarose (as used in this study) and/or the appropriate immunosorbent. All other biochemical and molecular biological methods (isolation of genomic and plasmid DNAs, electrophoresis of nucleic acids and proteins, PCR, isolation of DNA fragments from gels and their cloning in plasmid vectors, extraction of plasmid DNA from yeasts, Western blotting, etc.) were applied according to the recommendations of two currently classic laboratory manuals (Sambrook *et al*., 1989; Ausubel *et al*., 2009). Enzymatic manipulations with nucleic acids were performed using enzymes from New England Biolabs (United States) and Fermentas (Lithuania).

Bioinformatic methods for analysis of protein and nucleotide sequences. For phylogenetic and bioinformatic analysis of nucleotide and protein sequences of plants we used the most modern databases and approaches described in a monograph on plant bioinformatics (Edwards, 2007). Analysis of the secondary structure of the tobacco lnrRNA-547, the primary structure of which was established in this work, was performed using the software package RNAstructure tools (version 6.0.1).

RESULTS

Previously, we cloned and determined the primary structure of the adrenodoxin (ADX) related MFDXs' cDNAs of tobacco, tomato and *Digitalis purpurea* (Shematorova *et al.*, 2014) — the plant species which were used in study of the steroid hormone regulatory systems (Spivak *et al*., 2010; Shpakovski *et al*., 2017). Bioinformatic analysis of the structural conservation of mitochondrial type ferredoxins MFDXs in the world of plants has allowed to establish that the closest in structure to the corresponding protein of steroidogenesis in animals (adrenodoxin) are MFDXs from plants of the families Solanaceae and Scrophulariaceae (Fig. 1), in which we for the first time demonstrated the existence in plants of the very ancient progesterone hormonal system (Shpakovski *et al*., 2017).

We also confirmed that, as in the case of AtMFDXs of *Arabidopsis thaliana* (Ohta and Mizutani, 2004), the amino acid residues important for protein-protein interactions between [2Fe-2S]-FDX adrenodoxin (ADX) and FAD-containing flavoprotein adrenodoxin reductase (ADR) in the mammalian steroidogenesis system (Grinberg *et al*., 2000) are fully conserved in mitochondrial ferredoxins of tobacco (NtMFDX1 and NtMFDX2) and digitalis (DpMFDX2) (Fig. 2).

To obtain new information about physiological partners of these plant mitochondrial redox proteins, we used them as baits in screening of previously prepared comprehensive cDNA libraries of *Nicotiana tabacum* and *Digitalis purpurea* compatible with the YTH (yeast two-hybrid) system Interaction Trap (Golemis *et al*., 2009; Proshkin *et al*., 2011).

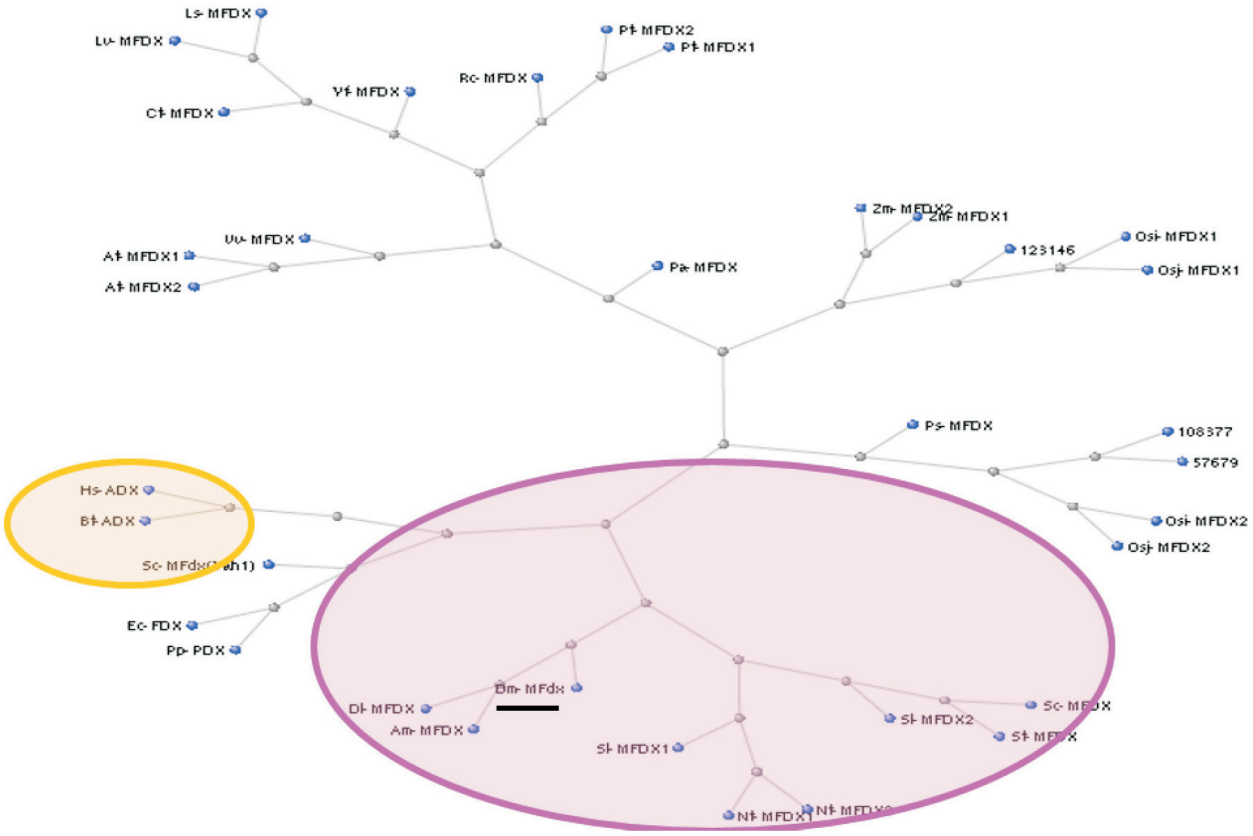

Fig. 1. Phylogenetic tree reflecting structural similarity of mitochondrial ferredoxins of plants from the families Solanaceae (Nt, Sc, Sl, St) and Scrophullariaceae (Am, Dl) with [2Fe-2S]-ferredoxins of Drosophila (Dm-MFdx; underlined by a greasy black line), yeast, bacteria and adrenodoxins of bull (Bt-Adx) and human (Hs-Adx).

Species abbreviations: Am – *Antirrhinum majus*, At – *Arabidopsis thaliana*, Bt – *Bos taurus*, Ct – *Carthamus tinctorius*, Dl – *Digitalis lanata*, Dm – *Drosophila melanogaster*, Ec – *Escherichia coli*, Hs – *Homo sapiens*, Ls – *Lactuca serriola*, Lv – *Lactuca virosa*, Nt – *Nicotiana tabacum*, Osi – *Oryza sativa* (Indica Group), Osj – *Oryza sativa* (japonica cultivar-group), Pa – *Persea americana*, Pp – *Pseudomonas putida*, Ps – *Picea sitchensis*, Pt – *Populus trichocarpa*, Rc – *Ricinus communis*, Sce – *Saccharomyces cerevisiae*, Sc – *Solanum chacoense*, Sl – *Solanum lycopersicum*, St – *Solanum tuberosum*, Vv – *Vitis vinifera*, Yf – *Yucca filamentosa*, Zm – *Zea mays*.

Fig. 2. Comparison of primary structures of mitochondrial ferredoxins of tobacco NtMFDX1 (Nt-mF1) and NtMFDX2 (Nt-mF2) and mitochondrial ferredoxin of *Digitalis purpurea* L. DpMFDX2 (Dp-mF) with amino acids sequences of bovine (Bt-Adx) and human (Hs-Adx) adrenodoxins. Positions of three specific amino acids changes in DpMFDX2 (Dp-mF) in comparison with DlMFDX1 (Dl on Fig. 1) sequence of *Digitalis lanata* (GenBank Accession Number AJ550155) are given in magenta.

Only one protein partner was found in this search for DpMFDX2 — in the proteome of *Digitalis purpurea* this ferredoxin interacts with the enzyme involved in biosynthesis of thiazole THI1 (Fig. 3), participating in the synthesis of the thiazole ring, and in its further condensation with a pyrimidine group to form thiamine pyrophosphate (vitamin B1). In plants, the synthesis of vitamin B1 occurs in both chloroplasts and mitochondria, and each of the organelles has its own special isoform of the THI1 protein, which is formed as a result of the differential use of two alternative ATG start codons of translation (Chabregas *et al.*, 2003). As seen from the primary structure of the corresponding cDNA, a mitochondrial type THI1 enzyme of *Digitalis purpurea* was identified in our search. The basis of the reac-

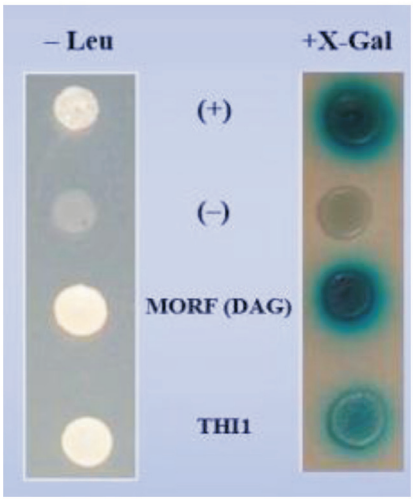

Fig. 3. Search for protein partners of mitochondrial NtMFDX1 ferredoxin of tobacco (interacts with protein MORF9 [DAG]) and mitochondrial ferredoxin DpMFDX2 of *Digitalis purpurea* L. (interacts with protein THI1) — activation of *LEU2* and *LacZ* reporter genes in yeast two-hybrid (YTH) Interaction Trap system tests.

tion catalysed by THI1 is the inclusion of a sulphur atom captured from cysteine by cysteine desulphurase IscS (in bacteria: Godoi *et al*., 2006) or Nfs1 (in eukaryotes). In this sense, the reaction catalysed by THI1 reminiscents the last stage of the synthesis of vitamin B8 (conversion of dethiobiotin in biotin) catalysed by biotin BIO2 (Picciocchi *et al*., 2001; 2003), which so far is the only currently known and rigorously proven function of plant MFDXs (Fig. 4).

In the case of *Nicotiana tabacum* mitochondrial ferredoxins we found that both NtMFDX1 and NtMFDX2 interact strongly with mitochondrial protein MORF9 (Fig. 3 and Fig. 5) and rather weakly with long (547 nt) non-coding RNA of a new type, which, on the one hand, binds to RNA polymerase II and stimulates transcription, and on the other, regulates (both positively and negatively) a number of components of the respiratory chain of plant mitochondria, in particular Nad1 and Nad5 proteins of complex I and the protein synthesis system of cytochrome c CMF (Fig. 6). The observed interactions of tobacco NtMFDX1 and NtMFDX2 with MORF9 protein suggest their participation in the processing (splicing, editing) of mitochondrial mRNA (please, see also the Discussion section).

The presence of adrenodoxin-like plant ferredoxins MFDX1 (At4g21090) = Adx1 (Picciocchi *et al*., 2001; 2003) and MFDX2 (at4g05450) = Adx2 (Picciocchi *et al*., 2003) in the mitochondria of *Arabidopsis thaliana*, along with another small ferredoxin, FD3 (At2g27510), has been previously proven (Salvato *et al*., 2014). Another study (Ohta and Mizutani, 2004) and our own data on the primary structure of MFDX1 and MFDX2 ferredoxins of tobacco, tomato and digitalis (see for example, Fig. 2) indicate that all amino acid residues of adrenodoxin, critical for its interaction with cytochrome P450scc, are strictly conserved in plants MFDX1 and MFDX2. To confirm the possible interaction of plant MFDXs with the mammalian cytochrome CYP11A1 (P450scc) in our previously generated transgenic plants efficiently expressing *CYP11A1* cDNA, we analysed the presence of different forms of P450scc in microsomal and mitochondrial fractions of the transgenic tomato cells (Fig. 7). Although apoenzyme (pre-P450ssc) was present both in microsomal and mitochondrial fractions of the transgenic plant cells, the mature, active P450ssc form was found only in mitochondrial fractions (Fig. 7). The results obtained indicate that in transgenic *CYP11A1* plants only the mature cytochrome P450ssc is transferred into mitochondria. These data correspond well with previous *in vitro* studies (Luzikov *et al*., 1994) and our own data about weak interactions of appropriate fragments of plant MFDXs and CYP11A1 in a yeast two-hybrid system (Shematorova *et al.*, 2018).

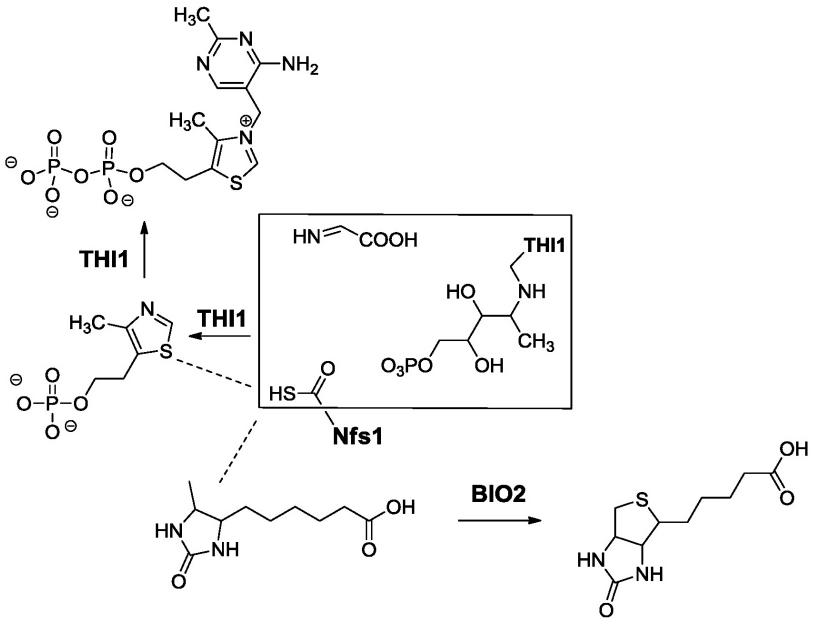

Fig. 4. Resemblance of the reaction of biosynthesis of thiazole (Godoi *et al*., 2006 – in bacteria; in eukaryotic organisms the protein THI1 performs the function of bacterial proteins ThiG and ThiE, up to the conversion of thiamine phosphate into vitamin B1 or thiamine pyrophosphate, TPP) and the last stage in the biosynthesis of biotin (vitamin B8): the insertion of a sulphur atom between the non-activated methyl and methylene carbon atoms adjacent to the imidazolidone ring (Picciocchi *et al*., 2003).

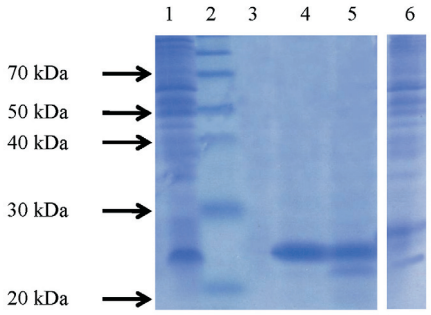

Fig. 5. Heterospecific expression of Nt-MORF9 (6xHis-MORF9 in pEXPR1-MORF9) and Nt-MFDX1 (from untaged pET21d-MFDX1) in *E. coli* and fractionation of separate and combined cellular extracts on TALON-agarose (Co 2+): Nt-MFDX1 coprecipitates with Nt-MORF9.

1 – lysate of bacterial cells containing plasmid pEXPR1-MORF9;

2 – protein molecular weight marker (Page Ruler^{TM} Broad Range Protein Ladder, Thermo Fisher Scientific);

3 – fractionation of cellular extracts with pEXPR1(control)+ pET21d-MFDX1;

4 – fractionation of cellular extracts with pEXPR1-MORF9 + pET21d (control);

5 – fractionation of cellular extracts with pEXPR1-MORF9 + pET21d-MFDX1;

6 – lysate of bacterial cells containing plasmid pET21d-MFDX1.

The identified range of biochemical reactions involving adrenodoxin related [2Fe-2S]-ferredoxins MFDX1 and MFDX2 of plant mitochondria are schematically shown in Fig. 8.

DISCUSSION

As their name implies (from fer – "iron" and redoxin – "reducing and oxidising" protein), ferredoxins are known primarily for their participation in the redox processes of cells: as components of electron transfer chains and electron carriers. Ferredoxins of plants containing iron-sulphur centers 2Fe-2S can be divided into two classes: plastid (localised and function in chloroplasts) and mitochondrial. When comparing the primary structures of these proteins with other well-studied proteins of the ferredoxin family (FDX), the former exhibit a clear homology (resemblance) with ferredoxin of the unicellular green algae *Chlamydomonas reinhardtii*, while the latter have a greater similarity with adrenodoxins (FDXs mitochondria of the adrenal cortex) of mammals or FDXs of other animals (Drosophila, nematode) and fungi (yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*). The first such analysis was published by Ohta and Mizutani (2004). They showed that in the model plant *Arabidopsis thaliana* there are nine genes encoding [2Fe-2S]-FDXs, six of which encode ferredoxin of chloroplasts and three encode mitochondrial type ferredoxins, of which the amino acid sequences of two show obvious similarity with adrenodoxin of mammals (bovine, human) (Figs. 1 and 2). In the same study, the Japanese authors were the first to detect the only ferredoxin reductase of mitochondrial type in plants (*Arabidopsis thaliana*), with similarity in primary structure to NADPH-adrenodoxin reductase. Compared to ferredoxins of chloroplasts, the functions of the mitochondrial ferredoxins are still poorly understood. Until now, it is not even clear how many types

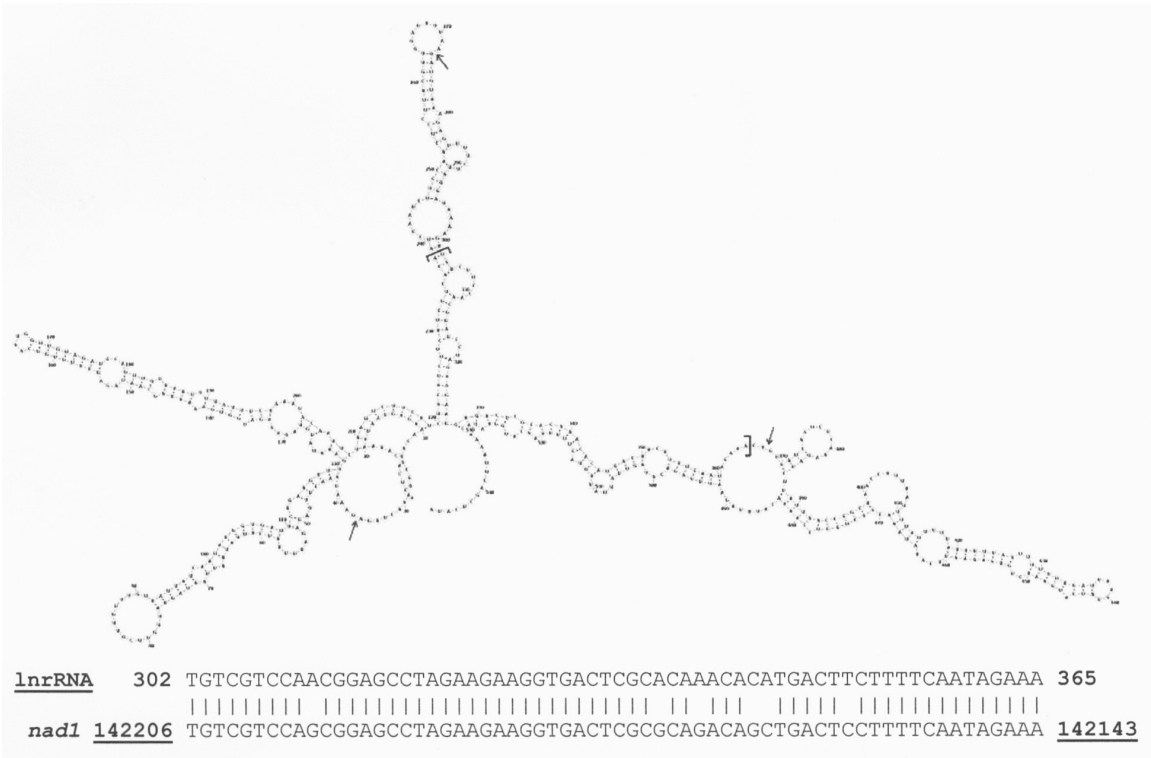

Fig. 6. The predicted optimal secondary structure of the selected long (547 nt) non-coding regulatory tobacco RNA (lncRNA-547), which interacts with mitochondrial ferredoxins MFDX1 and MFDX2. Red arrows show the areas of division (cutting) of the RNA primary structure into zones homologous to genes encoding such different components of the respiratory chain of plant mitochondria as Nad1, Nad5 and CcmF. Below is a specific homology with a fragment of the *nad1* gene (the corresponding part of the lncRNA-547 is highlighted on its secondary structure by blue brackets).

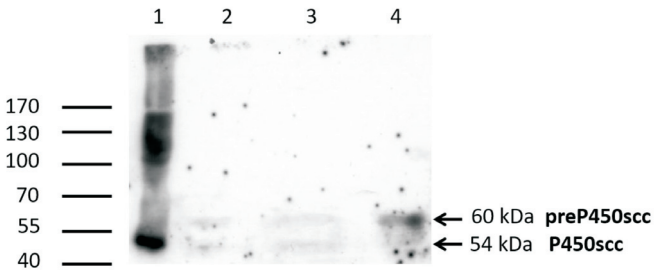

Fig. 7. Detection by Western blotting (10% PAAG-SDS, rabbit polyclonal Ab to human CYP11A1) of the predecessor protein (preP450scc) and the mature cytochrome CYP11A1 (P40scc) in different subcellular fractions isolated from the cells of calli cultures of some tomato TR (transgenic) lines expressing mammalian *CYP11A1* cDNA.

1 – lysate of the human ovary tissue;

2 – lysate of mitochondria of the TR tomato line No. 1;

3 – lysate of mitochondria of the TR tomato line No. 7;

4 – microsomal fraction (MS 15.07; microsomes).

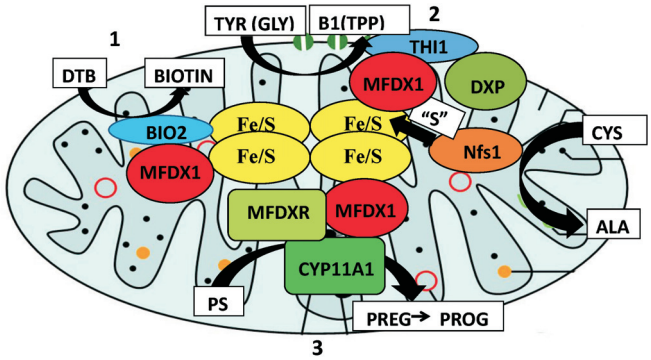

Fig. 8. The identified range of biochemical reactions involving adrenoxin-related [2Fe-2S]-ferredoxins of plant mitochondria MFDX1 (Adx1) and MFDX2: 1, the conversion of dethiobiotin into biotin (vitamin B8) [BIO2-complex] (Picciocchi *et al.*, 2003); 2, biosynthesis of thiazole and thiamin pyrophosphate (vitamin B1) [THI1-complex]; 3, the synthesis of pregnenolone (PREG) and then progesterone (PROG) from phytosterols (PS: β-sitosterol, campesterol, stigmasterol, cholesterol) [monoxygenase complex of cytochrome CYP11A1 in transgenic plants (Shpakovski *et al.*, 2017)]. AdxR (MFDXR) – homologue of adrenodoxin reductase in plant mitochondria, DXP – deoxyxylosophosphate synthase, Nfs 1 = IscS (in prokaryotes) – cysteine desulfurase.

of different mitochondrial ferredoxins exist and how many of them perform functions in these organelles of plant cells. To date, using proteomic methods only three proteins of the ferredoxin type [2Fe-2s] have been identified among the ~2000 mitochondrial proteins, two of which have structural similarity with mammalian adrenodoxins — exactly the same proteins that were originally described by Ohta and Mizutani (2004). These proteins were initially even named Adx1 (Picciocchi *et al.*, 2001) and Adx2 (Picciocchi *et al.*, 2003), but that is probably not really justified because they do not exhibit exceptional (extraordinary) homology (Fig. 2) and have specific (already clearly diversified compared to adrenodoxin) functions. Only one, and, apparently, not the main (and auxiliary) function that is established presently for these specific redox plant proteins is participation in the synthesis of vitamin B8 (biotin) together with BIO2 synthase (Picciocchi *et al.*, 2003).

Our new data on the interaction of these proteins with other components of the plant proteome show that in addition to participation in the electron transport chain of mitochondria (complexes I, II or III), these iron-containing [2Fe-2S] proteins are involved in the biosynthesis of the thiazole ring of vitamin B1 (thiamine pyrophosphate), editing of mitochondrial RNA, the correct formation of heme *A* in the cytochrome *c*, and probably even play an important role in the anterograde (from core to organelles) and retrograde (from organelles to nucleus) regulation of complexes I-IV of the respiratory chain of plant mitochondria.

Even with incomplete screening of the tobacco YTH cDNA library, we identified 17 clones (10 in the case of MFDX1 and 7 in the case of MFDX2) with MORF9 cDNA insertion, four of which are independent (have inserts with different primary structure). Participation of MORF proteins in RNA editing in the organelles of plants (in chloroplasts and mitochondria) has recently been shown (Takenaka *et al.*, 2012; Takenaka *et al.*, 2013). However, the enzyme that carries out the deamination of C (citidine) is still not identified (Castande and Araya 2011; Takenaka *et al.*, 2013). The transfer of electrons in deamination reactions is not in doubt, and therefore ferredoxins may well participate in this process in the form of auxiliary (accessory) helper proteins. Perhaps it is through the partners of our ferredoxins that it will be possible to trace (to detect) the editing enzyme that deaminates cytidines in plant mitochondria. In this respect, the crystal structure of the protein CDAT8 (Cytidine deaminase acting on tRNA base C8) performing C–>U editing in 30 different tRNAs of archaebacterial (archaea) *Methanopyrus kandleri* seems particularly interesting. CDAT8 consists of three domains: N-terminal cytidine-deaminase, Central ferredoxin-like (FLD) and C-terminal THUMP (RNA-binding domain). The modular organisation of CDAT8 suggests that the enzyme was formed by merging different domains. In the crystal structure, CDAT8 forms a dimer in which the cytidine-deaminase domains are located "head to head". The long linker connects to the tRNA-binding THUMP and ferredoxin-like (FLD) domains (Randau *et al.*, 2009; see also Haag *et al.*, 2017). This work clearly shows that the ferredoxin module is naturally used for interaction (binding) with RNA and for its subsequent editing (or at least creating conditions for the RNA deamination reaction). It is significant that the tandem of THUMP and FLD domains catalyses the binding of tRNA in other enzymes, including ThiI, responsible for the thiolation of the base U8 into 4-thiouridine. Strikingly, this enzyme involves sulphur atoms from the same Thi-cluster (thiC, thiD, thiG, thiH, thiO, thiL, etc. — in bacteria, THI1, THI4, THI5 — in plants and yeast) that was described above when discussing thiazole synthase THI1 (thiazole ring synthesis enzyme).

Finally, interaction of MFDX1 and MFDX2 with the long non-coding regulatory RNA that we have found may shed light on the long-anticipated function of ferredoxins in plant organelles in anterograde (nucleus to organelle) and retro-

grade (organelle to nucleus) signalling (see, e.g., Burch-Smith *et al*., 2011; Woodson *et al*., 2011; Ng *et al*., 2013). In addition, since the proteins of the mitochondrial oxidative phosphorylation system (for example, complexes III and IV of the respiratory chain or ATP synthase) are encoded by both nuclear (mainly) and mitochondrial genes, strict coordination of the biosynthesis of these proteins is necessary. Recently, it was shown that such synchronisation (ensuring the synthesis of approximately the same number of heterogeneous subunits by cytoplasmic and mitochondrial ribosomes) occurs mainly at the level of translation, not transcription (Couvillion *et al*., 2016). lnrRNA-547 detected in the complex with adrenoxin-like mitochondrial tobacco ferredoxins can participate in this "translational" coordination, where MFDX1 and MFDX2, which are synthesised in the cytoplasm but function in mitochondria, could perform the role of some kind of sensors for the particular state of oxidative phosphorylation system of the cell.

Although direct interactions of mitochondrial ferredoxins with cytochromes P450 of plants participating in the biogenesis of plant steroids have not yet been found, new data have been obtained in favour of the participation of adrenodoxin-like [2Fe-2S] ferredoxins in the functioning of the very ancient but only recently discovered 'progesterone' system of steroid hormonal regulation in higher plants.

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JAUNIZPĒTĪTĀ MIJIEDARBĪBA STARP ADRENODOKSĪN-SAISTĪTIEM [2Fe-2S] AUGU FERREDOKSĪNIEM MFDX1 UN MFDX NORĀDA UZ IzdarījuTO IESAISTĪŠANU PLAŠĀ FUNKCIJU KLĀSTĀ AUGU MITOHONDRIJOS

Ar ģenētiskām un bioķīmiskām metodēm, izmantojot kā modeļaugus *Nicotiana tabacum* L. un *Digitalis purpurea* L., tika pētīta nesen aprakstīto ferredoksīnu MFDX1 un MFDX iesaiste bioķīmiskos procesos mitohondrijos. Parādīts, ka šie proteīni piedalās steroīdu hormonālā regulācijā augstākiem augiem.