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Recombinant *Arabidopsis* WHY2 protein binds unspecifically to single-stranded DNA and is phosphorylated by mitochondrial protein kinases

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Abstract

Whirly proteins comprise a unique family of plant-specific DNA-binding proteins which are localized in both the nucleus and organelles. We have obtained a recombinant AtWHY2 protein, a mitochondrially targeted member of the *Arabidopsis* Whirly family, by the heterologous expression of *WHY2* gene in *E. coli* and subsequent purification. The purified protein bound efficiently to single-stranded DNA probes *in vitro*, but failed to bind double-stranded DNA probes. The binding of the AtWHY2 to single-stranded DNA probes did not depend on DNA sequence. It bound with similar efficiency to mitochondrial gene promoter regions, the protein-encoding part of *COB* gene lacking regulatory elements, and the nuclear elicitor response element. We have also demonstrated the phosphorylation of the AtWHY2 protein *in vitro* by mitochondrial protein kinases from maize and *Arabidopsis*. This finding suggests that activity of AtWHY2 and other Whirly family members could be regulated by phosphorylation that would be an interesting subject for further research.

Keywords: Whirly; *Arabidopsis thaliana*; DNA-binding proteins; mitochondrial genome; phosphoproteins.
Abbreviations: ERE - elicitor response element; GFP - green fluorescent protein; RT-PCR - reverse transcription polymerase chain reaction; SDS-PAGE - sodium dodecyl sulfate polyacrylamide gel electrophoresis; dsDNA - double-stranded DNA; ssDNA - single-stranded DNA.

Introduction

Proteins called «Whirly» are a small, recently discovered family of plant-specific DNA-binding proteins which have a number of intriguing features (Desveaux et al., 2005). The first described member of this family, StWHY1 protein from potato tubers, was initially characterized as a nuclear transcription factor involved in the activation of the *PR-10a* gene following pathogen-related stress (Desveaux et al., 2000). The protein was shown to bind specifically to the inverted repeat sequence of the elicitor response element (ERE) localized in the promoter region of this gene and some other stress-induced nuclear genes. Surprisingly, a transit peptide, responsible for the protein targeting into mitochondria or plastids, was later found in the amino acid sequence of all Whirlies (Desveaux et al., 2005; Krause et al., 2005). Moreover, all three members of the gene family in *Arabidopsis thaliana* were shown to be directed to either plastids or mitochondria in protoplasts transformed with the respective GFP fusion proteins (Krause et al., 2005). Further research showed that Whirly proteins play a specific role in organellar genetic processes. Thus, an involvement of the plastid-localized Whirlies in DNA recombination as well as in RNA metabolism was demonstrated (Prikryl et al., 2008; Marechal et al., 2009). Overexpression of the mitochondrially localized AtWHY2 protein in *Arabidopsis* altered the mtDNA copy number and the level of mitochondrial genes expression (Marechal et al., 2008). Recently, the role of Whirlies as modulators of DNA double-strand break repair through a microhomology-mediated break-induced replication pathway was revealed (Cappadocia et al., 2010; Cappadocia et al., 2012).

The unique ability of the same protein to take part in the regulation of activity of both organellar and nuclear genome made it possible to speculate about Whirlies participation in coordination of nuclear and mitochondrial or chloroplast gene expression via protein retranslocation from organelle to nucleus (Krause and Krupinska, 2009). The finding that barley WHY1 protein is located both in plastids and nucleus of the same cell (Grabowski et al., 2008) gives some support to this hypothesis. Despite growing interest towards Whirly proteins, many aspects of their regulation and functioning remain unexplored. The aim of the present work was to obtain the *Arabidopsis* recombinant WHY2 protein and to investigate the specificity of its binding to promoter regions of mitochondrial genes, as well as to study the possibility that this protein is phosphorylated by mitochondrial protein kinases. As a result, we have shown that the AtWHY2 protein is relatively unspecific with respect to its binding to various ssDNA probes *in vitro* but is subjected to phosphorylation by the mitochondrial protein kinases.

Results and discussion

Analysis of DNA binding activity of the recombinant WHY2 in vitro

We obtained the recombinant WHY2 protein in preparative quantities (1 mg) by the heterologous expression of *Arabidopsis WHY2* gene in *E. coli* cells and subsequent purification. The purified protein does not contain a mitochondrial transit peptide and its sequence is identical to

that of the putative mitochondrially localized native protein. According to SDS-PAGE analysis, the purified protein was homogeneous (Fig. 2b) and the molecular weight of the identified polypeptide was approximately 23 kDa, which corresponds to the size predicted on the basis of AtWHY2 amino acid sequence. The purified recombinant protein bound to all single-stranded DNA fragments used as probes for electrophoretic mobility shift assay (Fig. 1). At the same time, protein binding to double-stranded DNA probe with identical sequence was completely absent. This is consistent with previous observations showing that the binding of different Whirly family members is restricted mainly to ssDNA (Desveaux et al., 2005). The degree of recombinant AtWHY2 binding to ssDNA probes did not depend on their sequence. Both mitochondrial promoter regions carrying various consensus elements and a fragment of the *COB* gene, which did not contain any regulatory elements, proved to bind AtWHY2 to the same extent. Interestingly, the protein also bound with the similar intensity to the nuclear ERE sequence, which was described earlier as a specific site for the StWHY1 protein binding (Desveaux et al., 2000). Therefore, we conclude that the purified AtWHY2 shows features of sequence-unspecific ssDNA binding protein. Our data on the *in vitro* WHY2 binding to DNA correlate with the results of the study done with *Arabidopsis* transgenic plants overexpressing WHY2. The data of immunoprecipitation assays of the WHY2-DNA complexes in crude mitochondrial extracts demonstrate that the protein does interact with mtDNA *in vivo* and that intensity of this interaction is similar for different mitochondrial genome regions (Marechal et al., 2008). It should be noted that the lack of specificity of the WHY2 interaction with the mitochondrial DNA *in vivo* and with any ssDNA fragments *in vitro* does not exclude the possibility for this protein to be involved, along with some additional protein factors, in specific regulation of nuclear genes activity *in vivo*.

WHY2 is phosphorylated by mitochondrial protein kinases

Protein phosphorylation plays significant role in regulation of transcription factors binding to DNA (Meshi et al., 1995). Several dozens of polypeptides are phosphorylated in plant mitochondria with the help of specific protein kinases (Struglics et al., 2000; Bykova et al., 2003; Juszczuk et al., 2007). Protein phosphorylation participates in controlling binding of the protein factors to mtDNA (Tarasenko et al., 2008a). Protein kinases are also involved in transduction of signals from mitochondria to nucleus which causes alterations in nuclear-encoded mitochondrial protein expression (Tarasenko et al., 2009). Nevertheless, the precise protein targets subjected to phosphorylation in plant mitochondria remain largely unidentified. We studied the possibility of phosphorylation of the recombinant AtWHY2 by plant mitochondrial protein kinases. Extract of *Arabidopsis* mitochondria as well as partially purified sample containing unidentified maize mitochondrial protein kinases were used as sources of protein kinase activities (Subota et al., 2010). Fig.2 shows that polypeptide corresponding to WHY2 is phosphorylated in presence of both crude mitochondrial extract and partially purified protein kinases. We conclude that the WHY2 protein is an effective substrate for protein kinases localized in mitochondria. As far as we know, this is the first direct proof that a member of the Whirly family is a phosphoprotein. The possible involvement of phosphorylation in the activity regulation of one of the Whirlies has been reported earlier (Subramaniam et al., 1997). DNA binding capacity of the

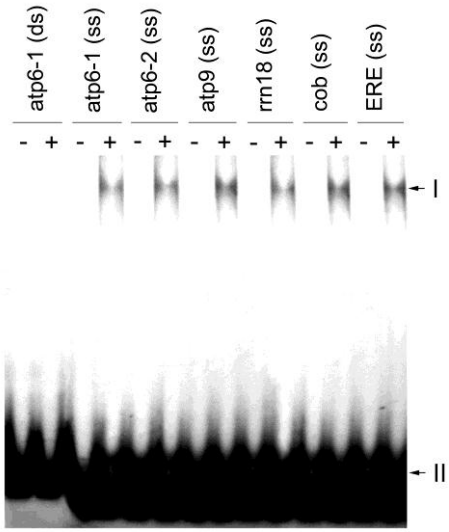

Fig 1. Purified recombinant AtWHY2 protein binds selectively to ssDNA, but does not demonstrate specificity with respect to the nucleotide sequence of the DNA probe. (I) DNA-protein complex position, (II) position of unbound radioactively labelled probe. (-) and (+) stand for samples without and with the addition of WHY2 protein, respectively. atp6-1, atp6-2, atp9, rrn18 correspond to DNA probes containing promoter regions of mitochondrial genes *ATP6*, *ATP9* and *RRN18*; cob corresponds to DNA probe containing sequence of *COB* gene fragment; ERE corresponds to nuclear elicitor response element. (ss) and (ds) indicate the single-stranded DNA probe and double-stranded DNA probe, respectively.

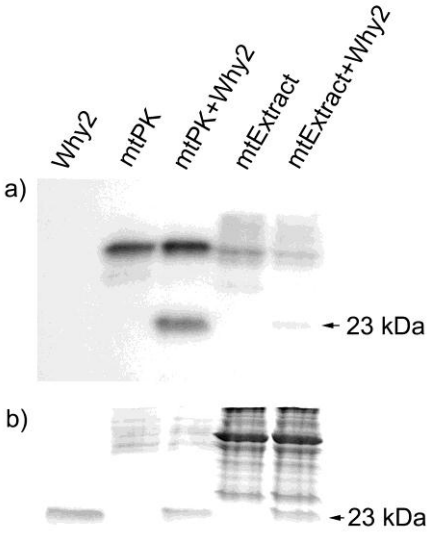

Fig 2. AtWHY2 protein is phosphorylated *in vitro* by mitochondrial protein kinases. a. – Autoradiograph of phosphoproteins separated by SDS-PAGE. b. – Polypeptide composition of protein samples. Following fractionation by SDS-PAGE proteins were stained with Coomassie R-250. mtPK– chromatographic fractions containing mitochondrial protein kinases from maize seedlings; mtExtract – extract of *Arabidopsis* mitochondria. Arrows indicate the position of 23 kDa band which corresponds to recombinant AtWHY2 protein.

StWHY1 was inhibited when potato tissues were treated with protein kinase inhibitors, indicating that phosphorylation could be involved in Whirly activation. It should be noted that StWHY1 has rather limited sequence homology with AtWHY2 and according to phylogenetic analysis belongs to another large Whirly subfamily (Desveaux et al., 2005). Therefore, phosphorylation may be assumed to be a common regulatory mechanism throughout the Whirly family.

Materials and methods

Plant Material

Seeds of *Arabidopsis thaliana* (L.) Heynh (ecotype Columbia) were purchased from Nottingham *Arabidopsis* Stock Centre (UK). Vertically mounted Petri dishes with 1/2 Murashige and Skoog (MS) salts and 0.8 % Phytagel were used to grow seedlings. Plants were grown at temperature of 23 ºC, 16-h photoperiod and irradiance of 100 μmol m^{-2} s^{-1}. Suspension cell culture was obtained from 7-d-old *Arabidopsis* seedlings and was maintained as described (Tarasenko et al., 2012).

Cloning and purification of recombinant AtWHY2 protein

The sequence of the *Arabidopsis WHY2* gene (GenBank accession number NM_105795) was used to generate primers 5'-CGTGCCATGGCAAGCTGGTCA-3' and 5'-CCCTCATCAGATCTTTTATCCCACT-3'. Using this pair of primers, the cDNA of *AtWHY2* gene lacking the sequence of the transit peptide was obtained. The total RNA extracted from 14-days old *Arabidopsis* plants was used as a template. RNA extraction, cDNA synthesis, RT-PCR, and cDNA sequencing were carried out as earlier described (Tarasenko et al., 2008b). The amplified fragment of the cDNA treated with the restriction endonucleases *Nco*I and *Bgl*II was cloned at relevant sites in the expression vector pQE60 (Qiagen, Valencia, CA, USA). Transformation of the *Escherichia coli* XL1-Blue cells (Fermentas, Vilnius, Lithuania) was done using the TransformAid (Fermentas). Induction of expression and purification of recombinant protein from 1 L of overnight bacterial culture on columns with Ni-NT-agarose (Qiagen) was performed according to the manufacturer's instructions. Eluted proteins were concentrated for 2 h against PEG6000 and dialyzed against the buffer containing 25 mM Tris-HCl, pH 7.8, 50 mM KCl, 0.5 mM EDTA, 10% glycerol, 14 mM 2-mercaptoethanol, 0.5 mM PMSF. Protein concentration was determined with Quant-iT Protein Assay Kit (Invitrogen, Carlsbad, CA, USA).

DNA-binding activity analysis

To measure the DNA-binding activity we used the DNA probes corresponding to promoter regions of mitochondrial *Arabidopsis* genes containing various consensus sequence elements and transcribed by two different phage-type RNA polymerases RpoTm and RpoTmp (Kühn et al., 2007): atp6-1 – 5'-GCCAATAATACGTATATAAGAAGAG-3'; atp9 – 5'-CTATCAATTTCATAAGAGAAGACGA-3'; rrn18 – 5'-AGTGGAATTGAATAAGAGAAGAAAG-3'; atp6-2 – 5'-TCTTGAATTAAGTATATAGAAAAGA-3'. Each of these probes spans the region from -18 to +6 relative to the transcription start site. The probe bearing a fragment of the protein-encoding part of the mitochondrial gene *COB* - 5'-TTCAACAGCGTAGAACACATTATGA-3', which lacks any regulatory elements, was also used. Probe containing ERE sequence (5'-CATTTTTGACATTTGTGTCATTTTAT-3'), which was shown to be necessary for the potato StWHY1 protein binding to promoter region of gene *PR-10a* (Desveaux et al., 2000) was used as a positive control. DNA fragments were labeled with polynucleotide kinase T4 (Promega, Madison, WI, USA) and 20 μCi of (γ-^{32}P) ATP (IRM, Moscow, Russia) per one sample. The reaction mixture was incubated for 40 minutes at 37 ^{o}C. The labeled probe was purified from unincorporated nucleotides using the Nucleospin Extract columns (Qiagen). DNA-binding activity was determined by electrophoretic mobility shift assay as described (Tarasenko et al., 2008a). Protein samples were incubated in a buffer containing 60 mM KCl, 1 mM EDTA, 10% glycerol, 20 mM Tris-HCl, pH 7.8, and a radiolabeled probe (15000 cpm, 100 fmol) at 20^{o}C for 20 min. The samples were loaded onto 5% polyacrylamide gel and run in TBE buffer (2 mM EDTA and 50 mM Tris-borate, pH 8.3) at 4 ^{o}C and 200 V for 1.5 h. The gel was fixed in 10% acetic acid for 15 min, dried and radioautographed.

Isolation of mitochondrial protein kinases

Arabidopsis mitochondria were isolated from suspension culture cells according to Sabar et al., 2005. Maize mitochondria were isolated from maize seedlings according to Subota et al., 2007. Maize seedlings were homogenized in isolation medium containing 0.25 M sucrose, 18 mM KH_2PO_4, pH 8.2, 5 mM EDTA, 10 mM KCl, 1 mM $MgCl_2$, 10 mM 2-mercaptoethanol, and 0.2% BSA. The homogenate was filtered through two layers of Miracloth. Nuclei and large cell fragments were separated by centrifugation (5 min, 5000 *g*). Mitochondria were sedimented by centrifugation at 15000 *g* for 5 min. The pellet was resuspended in washing medium (isolation medium devoid of 2-mercaptoethanol, pH 7.2) and centrifuged at 15000 *g* for 5 min. After resuspending the pellet in 5 ml of washing medium, mitochondria were purified on a discontinuous sucrose gradient (0.4, 0.8, and 1.2 M) by 30-min centrifugation at 5000 g. Mitochondria were resedimented at 15000 *g* for 10 min. The pelleted mitochondria were resuspended in the lysis buffer containing 25 mM Tris–HCl, pH 7.8, 600 mM KCl, 1 mM EDTA, 10% glycerol, 14 mM 2-mercaptoethanol, 0.5 mM PMSF, and 0.1% BSA. Triton X-100 (10%) was added to a final concentration of 0.75%, and the mixture was incubated at 4°C for 10 min and centrifuged at 20000 *g* for 15 min. The supernatant was collected and dialyzed against the buffer used at further stages of purification. Chromatographic purification of protein kinases was performed on columns with Sephadex G-200 (Pharmacia, Uppsala, Sweden) and DEAE-Toyopearl (Toyo Soda, Tokyo, Japan) as described (Subota et al., 2010).

Protein phosphorylation in vitro assay

Protein phosphorylation was carried out according to Subota et al., 2010. The mitochondrial extract or the chromatografic fractions were resuspended in buffer containing 0.3 M sucrose, 50 mM Hepes–KOH, pH 7.5, 5 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 0.2 mM (γ-^{32}P) ATP (0.4–1.0 Ci/mM) (IRM, Russia). Samples were incubated for 15 min at room temperature. The reaction was stopped by adding the sample buffer containing 2% SDS, 10% sucrose, 5% 2-mercaptoethanol, and 60 mM Tris–HCl, pH 6.8. The mixture was heated at 100°C for 2 min. Protein samples were analyzed by SDS-PAGE in 10% gel. The gel was exposed with Kodak X-ray film for at least 7 days.

Conclusion

We have obtained recombinant AtWHY2 protein and have confirmed *in vitro* that its binding to DNA does not depend on DNA sequence. This data suggests that AtWHY2 functions in mitochondria as sequence-unspecific ssDNA-binding protein participating in DNA repair or recombination rather than in transcription initiation. It is unclear at the moment if AtWHY2 could play a role of transcription factor in nuclei as it was shown for StWHY1. If this is the case, additional protein factors apparently would confer specificity of AtWHY2 binding to nuclear gene promoter regions. Further research is needed to address the question of AtWHY2 functions in nucleus. We have also demonstrated the phosphorylation of the AtWHY2 protein by mitochondrial protein kinases. This finding suggests the possibility of AtWHY2 activity to be regulated by phosphorylation. It would be interesting for further research to study the effect of phosphorylation on activity of AtWHY2 and other Whirly family members.

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