Ph.D. thesis

Arabidopsis PPR40 connects abiotic stress responses to mitochondrial electron transport

Zsigmond Laura

Supervisor: Dr. Szabados László

Arabidopsis Molecular Genetic Group
Institute of Plant Biology

Biological Research Center
Hungarian Academy of Sciences

University of Szeged

Szeged
2008

Background

Adaptation of plants to environmental changes has influence on numerous metabolic pathways, including changes in photosynthesis, respiration, metabolite assimilation and catabolism. Mitochondria are in the centre of regulation of cellular energy homeostasis and redox balance and integrate lot of metabolic pathways (e.g. Krebs cycle) that are important in adaptive responses to extreme environmental conditions. Respiration is the core process of mitochondrial metabolism; during this procedure large amount of free energy is released from the oxidative phosphorylation reactions of electron transport system, and it used for ATP production. The electron transport chain of plant mitochondria is composed of four respiratory complexes. Depending on the substrate, electrons are transported from Complex I (NADH dehydrogenase) and Complex II (succinate dehydrogenase) through ubiquinon and Complex III (cytochrome c reductase) to cytochrome c and to Complex IV (cytochrome c oxidase), which produces water. In the energy conserving pathway ATP is generated by Complex V (ATP synthase), which is connected to the electron transport system tight. When the electron transport in the cytochrome c pathway is blocked (stress conditions, specific inhibitors), alternative oxidases (AOX) help to maintain the electron flux and functional Krebs cycle.

Plant mitochondrial electron transport is also important to neutralize the excess of reducing capacity of photosynthesis, preventing oxidative damage of thylakoid membranes and other cellular components. Reactive oxygen species (ROS) are produced in the mitochondrial electron transport chain, where Complex I and Complex III are major sites for ROS synthesis in the darkness and in non-green tissues. Mitochondrial electron transport is implicated in ROS production during different biotic and abiotic stresses. ROS can oxidize and damage cellular structures, macromolecules, nucleic acids, proteins and lipids. Besides being damaging agents, ROS are important signalling compounds

implicated in the control of plant development, adaptation to environmental stress conditions, defense and programmed cell death. In interaction with other signalling molecules (e.g., lipid signals, nitrogen oxide, calcium ions and plant hormones) ROS control protein stability and gene expression.

The pentatricopeptide repeat (PPR) protein family is particularly large in plants. In the *Arabidopsis* genome 441 putative *PPR* genes were identified. Although several *PPR* genes have been characterized in the last few years and it is known that PPR proteins are localized either in mitochondria or plastids, the biological function of most PPR genes is still unknown. The PPR protein family is characterized by 9-15 tandem arrays of pentatricopeptide repeats, which are composed of degenerate 35 amino acid units. PPR repeats form helical structures and are considered to be RNA binding motifs. PPR domains are related to tetratricopeptide repeats (TPR), which can participate protein-protein interactions suggesting that PPR domains may have similar functions also. PPR proteins are implicated in the regulation of organellar gene expression by controlling diverse aspects of organellar RNA metabolism, such as RNA splicing, editing, processing and translation. The PPR genes can influence diverse biological processes including cytoplasmic male sterility, circadian clock, seed development, and transcription and translation of plastid-encoded mRNAs and proteins. Embryo lethality, reduced fertility and dwarf phenotype was associated with several PPR gene mutations suggesting that they have important functions in the regulation of plant growth and development.

Aim of our study

In our laboratory we have identified an ABA hypersensitive T-DNA insertion *Arabidopsis thaliana* mutant (*ppr40-1*) which showed semi-dwarf growth. We would have liked to characterize the function of the *PPR40* gene

affected by the mutation with the detailed examination of this mutant. For the characterisation we had to answer for the following questions:

- Does the mutant show sensitivity against different types of abiotic stresses?
- Does the T-DNA insertion result in inactivation of *PPR40* gene, and is there connection among stress responses and gene inactivation?
- Could the changed phenotype of the mutant be restored by overexpressing of *PPR40*?
- What kind of intracellular localisation does the PPR40 protein have?
- Is there any relationship between protein localisation and stress sensitivity of the *ppr40-1* mutant?

Materials and Methods

- Plant materials and growth conditions
- Identification and characterization of insertion mutations
- RNA isolation and analysis
- Preparation of mitochondria, protein isolation and detection
- Mitochondrial protein complexes separation by sucrose gradient centrifugation and 2D Blue-Native/SDS PAGE
- Protein identification by mass spectrometry
- Immunocytology
- Measurement of H_2O_2 production
- Lipid peroxidation assay
- Determination of ABA content
- Enzyme assays (APX, SOD, cytochrome c oxidase)
- Stomata opening assay
- Measurement of ascorbate consumption and respiration in mitochondria

Results and Discussion

The *ppr40-1* mutant was identified from our T-DNA-tagged *Arabidopsis* mutant collection (Szabados et al., 2002). The mutant showed semi-dwarf growth and ABA hypersensitivity. Characterization of the *ppr40* mutation identified a tandem inverted T-DNA in the transcribed region of gene *At3g16890.* The T-DNA insertion was localized 311 bp downstream of the predicted ATG codon. In the SALK insertion mutant collection we characterized a second mutant allele (*ppr40-2*), in which the T-DNA insertion occurred at 852 bp downstream of the ATG. The predicted PPR40 protein has 14 PPR domains, arranged in 5 and 9 tandem repeats. The *ppr40-1* and *ppr40-2* mutants represented a strong and a weak allele, respectively, with clear difference in plant growth and ABA sensitivity: *ppr40-1* was 50% smaller, whereas the *ppr40-2* mutant was 20% smaller in seedling and rosetta stage in comparison to wild type; in the presence of 0.5 μM ABA only 20% of *ppr40-1* seeds germinated after 1 week compared to germination of 60% *ppr40-2* and 98% wild type seeds. Predicted N-terminal translation products in the *ppr40-1* allele terminated before the PPR repeats, while in the *ppr40-2* allele the predicted truncated protein carries five N-terminal PPR domains. The difference in the phenotypes of the two alleles suggest that the truncated protein with five N-terminal PPR domain, which is produced in the *ppr40-2* is partially functional, while the *ppr40-1* allele represents a real knockout mutation.

In order to characterize the physiological responses of the *ppr40-1* mutant to various environmental conditions and plant hormones, we performed seed germination and seedling growth assays. The *ppr40-1* proved to be sensitive to glucose, sucrose and NaCl during germination and root elongation, but had no difference in germination and growth of *ppr40-1* and wild type in response to changes of other environmental (continuous light and dark) and stress (heat-

shock and heavy metals) conditions. Except ABA, the *ppr40-1* mutant and wild type also showed similar responses to treatments with plant hormones.

To confirm the connection between the mutant phenotype and the T-DNA insertion mutation in the *At3g16890* gene, we performed the *PPR40-HA* gene construct and introduced into the *ppr40-1* mutant by *Agrobacterium*-mediated transformation. The phenotype of complemented *ppr40-1* plants was similar to wild type in all germination and seedling growth assays. Consequently, we confirmed that the *ppr40-1* mutant phenotype was indeed caused by the T-DNA insertion mutation.

To further analyze the biological function of this protein, we transformed the *PPR40-HA* gene construct into the wild type plants. Stress responses of the *PPR40-HA* overexpressing plants were tested in germination assays. The PPR-HA protein overexpressing transgenic lines showed insensitivity to toxic concentrations of NaCl, glucose, and ABA treatments at germination level. Improved germination of PPR40 overexpressing plants suggested that PPR40 protein may have important protective role in plants.

On the base of ABA sensitivity we examined the ABA induced stomatal closure and water loss of *ppr40-1* plants in a desiccation assay. We found that stomatal closure is more sensitive to ABA in the *ppr40-1* mutant compared to wild type. Water loss of the mutant leaves was significantly slower compared to wild type correlating with ABA sensitivity of stomatal closure of the *ppr40-1* mutant. To determine whether enhanced ABA sensitivity of *ppr40-1* correlates with either elevated ABA biosynthesis or alteration in signal transduction, we compared the free ABA concentrations in wild type and *ppr40-1* plants. We did not find notable difference between the mutant and wild type plant only as results of extreme stress conditions. This point to the fact that presumably the mutation has no direct connection to ABA biosynthesis. To study the transcript levels of a few stress and ABA induced transcription factors and other regulatory genes we performed a series of quantitative RT-PCR assays. The

expression data indicated that the *PPR40* function was not implicated in the primary control of ABA signalling.

Although the *ppr40-1* mutant is more sensitive to salt and osmotic stress, we found that it showed increased tolerance to methylglyoxalate. Methylglyoxalate is catabolized by the glyoxalase system, consisting of two enzymes, glyoxalase I (*GLX1*) and glyoxalase II (*GLX2*). We examined the expression of several *GLX1* (*GLX1-1, GLX1-2, GLX1-3*) and *GLX2* (*GLX2-1, GLX2-2*), and our results showed that glyoxalase genes have higher transcript levels under standard growth conditions in the *ppr40-1* mutant compared to wild type. Enhanced expression of glyoxalase genes appeared thus to account for the enhanced MG tolerance of the *ppr40-1* mutant.

In order to determine the intracellular localisation of PPR40 protein, we analysed PPR40-HA protein overexpressing plants and cell culture. The presence of PPR40-HA protein in different subcellular fractions was tested by western blotting. The PPR40-HA protein was detected in mitochondria but not in chloroplasts or in other organelles. Immunohistochemical detection has also verified the mitochondrial localization of the PPR40 protein. To search for possible function of PPR40, we investigated the association of PPR40-HA with protein complexes of mitochondrial electron transport system using sucrose gradient centrifugation and 2D Blue-Native/SDS polyacrylamide gel electrophoresis followed by detection of HA-epitope. In both cases we observed immunoblotting detected PPR40-HA in a protein complex of about 500 kDa. The size of this complex corresponded to that of Complex III of mitochondrial electron transport system. Composition of the PPR40-HA protein-associated protein complex was analyzed subsequently by mass spectrometry. The results of proteomics analysis thus confirmed that PPR40 was associated with Complex III. To compare wild type and *ppr40-1* mutant mitochondrial respiratory complexes and the subunits of Complex III we find no different in patterns thus the *ppr40-1* mutation does not affect the composition of Complex III.

Furthermore, the *ppr40-1* mutation did not appear to influence the transcript levels of genes coding for subunits of Complex III.

To examine whether PPR40 controls splicing or editing of mitochondrial mRNAs, we analysed the mitochondrial apocytochrome B (*cob*). *cob* is the only Complex III subunit, which is encoded by the mitochondrial genome and is expressed as a 5 kb transcript. We observed no difference in *cob* transcript size between mutant, wild type and complemented plants. To test the editing sites of *cob* mRNA, the full length *cob* cDNA was amplified and sequenced from RNA samples of wild type, mutant and complemented mutant plants. RNA editing did not change in the *ppr40-1* mutant.

Association of the PPR40 protein with Complex III in the mitochondrial electron transport system prompted us to test possible alterations in respiration and associated mitochondrial functions, such as consumption of different respiration substrates and generation of reactive oxygen species. Respiration was measured by oxygen consumption in intact mitochondria. When NADH (electron donor for Complex I) was used as respiratory substrate, oxygen consumption was 50% lower in the *ppr40-1* mitochondria compared to the wild type. When succinate (electron donor for Complex II) was added oxygen consumption 40% lower in the *ppr40-1* mitochondria. These data indicated that electron transport through Complex I and Complex II was greatly reduced in the mutant. To test the other function of the electron transport system, we used ascorbate as respiratory substrate for Complex IV to measure direct electron transport from this substrate to oxygen. We detected 2.5-3.0-fold higher oxygen consumption in *ppr40-1* mutant mitochondria compared to wild type. Furthermore, we observed that cytochrome c oxidase activity was about twice as high in the *ppr40-1* mutant than in wild type, and the ascorbate consumption was 25% higher in roots and 50% higher in cell culture of the *ppr40-1* mutant compared to wild type. These results showed that Complex IV is not only fully functional, but it works at a higher rate and ascorbate could at least partially

bypass the defect of electron transport in the *ppr40-1*. Reduced respiration rates in the *ppr40-1* mutant suggest that PPR40 protein is essential for the electron transport through Complex III.

In mitochondria Complex III was shown to be the principal source of reactive oxygen species (ROS). Inhibition of cytochrome c oxidase activity increases ROS generation and oxidative damage. In plants mitochondrial ROS production can be reduced by non-phosphorylating respiratory pathways, which includes alternative oxidases (AOX). AOX activity bypasses Complex III and Complex IV of the electron transport chain. Our measurements showed that the AOX activity was 40% higher in the *ppr40-1* mutant compared to wild type mitochondria. We also compared the stress-responsive *AOX1d* transcript levels in *ppr40-1* and wild type plants. In the *ppr40-1* mutant elevated transcript level of the *AOX1d* gene has been found, which clearly indicates activation of the non-phosphorylating respiratory pathways as consequence of the impeded Complex III function.

Since Complex I and Complex III are considered to be the main sources of ROS generated in mitochondria during oxidative respiration, we have tested ROS accumulation and effect of oxidative damage in *ppr40-1* mutant. Comparison of hydrogen peroxide production in mitochondria isolated from either roots or cultured cells of *ppr40-1* mutant and wild type indicated 30% higher H_2O_2 levels in *ppr40-1* mitochondria. Lipid peroxidation is a direct consequence of ROS damage and is therefore considered as major indication for ROS accumulation. We observed that the ratio of oxidized lipids was 20 to 25% higher in leaves of the *ppr40-1* mutant compared to wild type plants. Superoxide radicals are known to be generated by Complex III misfunction during stress and represent the most damaging ROS species. Superoxide radicals are converted to H_2O_2 by mitochondrial manganese-containing superoxide dismutase. We found that the SOD activity was 15% higher in leaves of the *ppr40-1* mutant compared to wild type plants. However, this difference was more pronounced when

MnSOD activity was measured in isolated mitochondria, which indicated 40% higher activity in *ppr40-1* than in wild type. The observed increase of mitochondrial MnSOD activity therefore suggested enhanced generation of superoxide radicals and subsequent hydrogen peroxide accumulation in the *ppr40-1* mutant, which was in fact detected in our previous assays. Enhanced H_2O_2 levels and lipid peroxidation in the *ppr40-1* mutant suggest that AOX activation cannot compensate completely the disturbed cytochrome c oxidase activity. In fact, the *ppr40-1* mutant shoved increased sensitivity to externally added hydrogen peroxide or the ROS generating herbicide, paraquat. The detoxification system of *ppr40-1* is probably overwhelmed by ROS, which was generated by the damaged mitochondrial electron transport and detoxification is therefore insufficient to reduce effectively the increased ROS levels during stress conditions. In the *ppr40-1* mutant mitochondria with reduced respiration may generate a permanent stress condition, with constitutive stress signals activating metabolic defences. Therefore, enhanced sensitivity of the *ppr40-1* mutant to high salinity probably also reflects an enhanced ROS damage during stress.

Summary

Here we describe a mitochondrial pentatricopeptide (PPR) domain protein, PPR40, which provides a signalling link between mitochondrial electron transport and regulation of stress and hormonal responses in *Arabidopsis thaliana*. Insertion mutations inactivating PPR40 result in semi-dwarf growth habit and enhanced sensitivity to salt, ABA and oxidative stress. Genetic complementation by overexpression of *PPR40* cDNA restores the *ppr40* mutant phenotype to wild type. The PPR40 protein is localized in the mitochondria and found in association with Complex III of the electron transport system. In the *ppr40-1* mutant the electron transport through Complex III is strongly reduced,

while Complex IV is functional, indicating that PPR40 is important for the ubiqinol-cytochrome c oxidoreductase activity of Complex III. Enhanced stress sensitivity of the *ppr40-1* mutant is accompanied by accumulation of reactive oxygen species, enhanced lipid peroxidation, higher SOD activity and altered activation of several stress responsive genes including the alternative oxidase *AOX1d*. These results suggest a close link between regulation of oxidative respiration and environmental adaptation in Arabidopsis.

Publications

Szabados L, Kovács I, Oberschall A, Ábrahám E, Kerekes I, **Zsigmond L**, Nagy R, Alvarado M, Krasovskaja I, Gál M, Berente A, Rédei GP, Haim AB, Koncz C (2002) Distribution of 1000 sequenced T-DNA tags in the Arabidopsis genome. Plant J 32: 233-242

Zsigmond L (2004) Characterisation of the ABA sensitivity influencing Arabidopsis PPR gene. Acta Biol Szeged 48:93

Zsigmond L, Rigó G, Szarka A, Székely Gy, Ötvös K, Darula Zs, Medzihradszky KF, Koncz Cs, Koncz Zs, and Szabados L (2008) Arabidopsis PPR40 connects abiotic stress responses to mitochondrial electron transport. Plant Physiol 2008 Feb 27 (Epub ahead of print)