Theses of PhD
(short version)

Isolation and Characterization of *Mhb1*, a Novel Non-symbiotic Hemoglobin From *Medicago sativa*

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Introduction

Plant hemoglobins are usually divided into three major groups on the basis of their common features. The group of symbiotic hemoglobins has been known since 1939. They can be found in leguminous plants and non-legumes living in symbiosis with nitrogen-fixing organisms. Their role is to provide oxygen to symbionts in tissues actively fixing nitrogen.

The most recently discovered (2001) plant hemoglobin group is called 2-on-2 hemoglobins. Their name implies a structural difference from the other two major groups, and this probably also means their different function. The so far only such a gene (*AHB3*) shows the highest homology to the truncated hemoglobins of microorganisms and it is down regulated by hypoxia.

Nonsymbiotic hemoglobins, termed also as "phytoglobins" belong to the third group. They are not only present in plants containing symbiotic hemoglobins, but also in other plant species e.g. *Arabidopsis*, barley, rice and *Trema tomentosa*. Furthermore, nonsymbiotic hemoglobins are regarded to be more ancestral as symbiotic hemoglobin formation was the result of nonsymbiotic hemoglobin gene duplication.

Since phytoglobins were discovered significantly later (1988) than symbiotic hemoglobins, we do not know much about their function. Their characteristics include higher affinity to oxygen than symbiotic hemoglobins have, their inducibility under low oxygen tensions which could mean an oxygen sensing function. All hypoxia-inducible nonsymbiotic hemoglobins belong to class 1 of nonsymbiotic hemoglobins. The so far only hemoglobin with close homology to phytoglobins that is induced upon cold stress and not by hypoxia is considered as a class 2 nonsymbiotic hemoglobin (*AHB2* from *Arabidopsis thaliana*).

Furthermore, high mRNA levels of phytoglobin were observed in the roots and rosette leaves of barley, young leaves, stems and roots of soybean, in rice leaves and roots and in *Arabidopsis thaliana* roots.

Recently published results have shown two major fields where phytoglobins can have functional importance. According to the first one, phytoglobins are induced upon decrease of intracellular ATP level. This decrease may be caused by hypoxia or elevated metabolic activity (*e.g.* cell division in the meristem). It has been shown that in a barley phytoglobin overproducing maize suspension culture grown under partial (5% O_2) hypoxia, the ATP level was about 30% higher than in non- transformed control suspension.

The other major phytoglobin function started to outline when studies appeared about the different roles of nitric oxide (NO) in plants, with particular respect to its function in plant disease resistance. NO, similarly to its activatory role in mammalian defense responses, is an important part of the plant disease resistance system. Application of NO donors to or overexpression of recombinant mammalian nitric oxide synthase gene in tobacco plants or cell suspensions induced the expression of the defence genes encoding the PR-1 protein and the enzyme phenylalanine-ammonia lyase. In plant defense responses it was also shown that cGMP and cyclic ADP-ribose act as second messengers of NO and they can act synergistically, just as reported in the case of animal cells.

NO can function both in animals and plants as a messenger of programmed cell death. It is also known that the NO-signalling pathway can either be cGMP-independent (such as the formation of peroxynitrite and nitrosylation) or cGMP-dependent in animals and plants. *E.g.* in animals NO could induce MAP kinase cascades leading to apoptosis. In plants, NO-induced cGMP synthesis is

necessary for NO-induced cell death (of cultured *Arabidopsis* cells) through the activation of a MAP kinase upon incubation with NO-donor compounds.

Furthermore, it became clear that some key effects of NO are mediated by interactions with hemoglobin in very distantly related organisms. It was speculated that phytoglobins could also have a similar role. Recent reports can support this hypothesis. During hypoxia NO production was observed in maize cell cultures and alfalfa root cultures. Similarly to the situation in bacteria, phytoglobins could help detoxify this compound. This was supported by results showing a greater amount of NO in transformed lines with reduced phytoglobin expression than in wild type or phytoglobin-overproducing lines. It is supposed that oxyhemoglobin oxidized NO to NO_3^- because nitrate is much less toxic for plants and it can be utilised easily. The direct binding of NO to heme (nitrosylhemoglobin) can also lower intracellular NO levels, and thus it can prevent cells from NO- induced apoptosis.

Aims of Study

Since little has been known about phytoglobins, the purpose of my work was the molecular and functional characterization of the novel *Mhb1* gene and the encoded phytoglobin protein. Two major steps have been taken to achieve this goal:

I. In the first step our aim was the molecular characterization of the *Mhb1* gene with particular respect to the following questions:
   1. In which alfalfa plant tissues is this gene active?
   2. Can this gene be activated if exposed to stress factors previously known to induce other nonsymbiotic hemoglobin genes?
   3. Does this gene have something to do with the earlier described change of metabolic activity of the cells?
   4. What other features of the *Mhb1* gene and the encoded protein can be found that could help determine its function?

II. In the second step my purpose was to construct transgenic plants overproducing the Mhb1 protein, and to perform a functional characterization. Basically, we intended to answer the following questions:
   1. Are there any phenotypic differences between transformant and non-transformant plants?
   2. How else is it possible to make distinction between transformant and non-transformant plants with respect to Mhb1 protein function?

Methods

A cell suspension culture of *Medicago sativa* ssp. *varia* (genotype A2, tetraploid) was maintained and used for different stress treatments, synchronization (including flow cytometric analysis) and preparation of a cDNA library.

Recombinant DNA work (gene cloning and transformation, isolation of plasmid DNA, genomic hybridization)

Isolation of total RNA, Northern blot analysis

Recombinant protein production in bacterial expression system, purification of the recombinant protein

Salicylic acid level measurement by HPLC-analysis

Reactive oxygen species determination by NBT-method

Results

In sequenced alfalfa cDNAs we have identified a 483 basepair long clone as *Mhb1*, a 160 amino acid long (18 kD) nonsymbiotic non-leghemoglobin. *Mhb1* is a full length clone and has all the features that are characteristic of plant hemoglobins. These features include strong homology to other nonsymbiotic hemoglobins (70-80%) and conserved amino acid residues including the plant globin motif.

To determine *Mhb1* gene copy number and to investigate if there are any cross-hybridizations to leghemoglobins or to other types of nonsymbiotic hemoglobins, Southern hybridization was performed to genomic DNA isolated from diploid *Medicago sativa* plants that were used earlier to construct the detailed genetic map of alfalfa. The result could show the alleles of a single gene, which locates on the 40-47 cM region of linkage group 4.

Northern analyses of total RNA samples obtained from various plant tissues grown under normal conditions have revealed observable *Mhb1* RNA level only in the *Medicago* roots.

We used Northern blot experiments to study the *Mhb1* gene expression after exposing cultured alfalfa cells to hypoxia and cold stress. In dedifferentiated alfalfa callus cells grown in the presence of 2,4-D and cytokinin the *Mhb1* gene showed low, basal level of transcription that was not influenced by cold stress treatment. In the same culture a significant change in the *Mhb1* RNA amount was detected as a result of the hypoxic treatment as early as 2 hours after the start of hypoxia, and this high level is maintained throughout the experiment (24 h). However, after exposing cultured cells to cold stress and hypoxia, respectively, the Mhb1 protein accumulation was the same as in cells grown under normal conditions.

To test if there is a cell cycle dependence of *Mhb1* gene transcription, we synchronized alfalfa cell suspension culture with hydroxyurea. *Mhb1* mRNA level changes at 10-12 hours show the induction of the *Mhb1* gene before mitosis.

We also determined changes in the metabolic activity of the cells during cell division. Respiration rate measurements on synchronized alfalfa cell suspension culture have been carried out. Three major changes of the respiration rate can be observed during the cell cycle. At 14 hours (before mitosis) there is a reproducibly detectable peak in the respiration rate (from 100% to 119.4%, where 100% is the respiration of an unsynchronized culture). Between 16 and 18 hours (during and right after mitosis) there is a decrease in respiration rate (from 119.4% to 109.4%). From 20 hours after removing hydroxyurea the (during G1 phase) the respiration rate starts to increase again until it reaches 149%, and it remains within this range until the end of the experiment (24h).

In order to determine the intracellular distribution of Mhb1 protein in alfalfa cells, immunoelectron microscopy was carried out. The vast majority of the labelling can be seen within the nucleus, with nucleoli virtually unlabelled. However, to a lower extent, cytoplasmic labelling is also observable. The immunogold localization of the Mhb1 protein in alfalfa root tip cells showed the same results.

We have generated transgenic tobacco plants constitutively overexpressing the alfalfa nonsymbiotic hemoglobin Mhb1 (using the CaMV 35S promoter). The presence of the *Mhb1* transgene product was checked using polyclonal antibody raised against a recombinant Mhb1 protein. Furthermore, we

could not detect any phenotypic differences between untreated transformant and control plants.

Similarly to adult plants, no phenotypic or growth rate difference could be seen between untreated transformed and non-transformed seedlings. However, a considerable retardation of germination could be observed both in the transformed and non-transformed seedlings grown in the presence of 300 μM SNP (NO-donor) compared to the untreated ones. The growth and development of transformed seedlings were less retarded by SNP-treatment. The Mhb1-overproducing seedlings developed cotyledons and radicules while the non-transformed seedlings formed only radicules. Furthermore, when tobacco seedlings were grown with 300 μM of inactivated SNP to test the role of unspecific SNP-components, significant retardation was observed regardless of the genotype.

However, the non-transformant seedlings treated with active SNP were significantly smaller than their counterparts treated with the inactivated SNP.

We have infiltrated 5 mM active and light-inactivated SNP solutions to adult transformant and control plant leaves, respectively. The light-inactivated SNP affected only weakly the treated leaves. However, comparing the damages on transformed and control leaves, we observe a significantly smaller necrotic area on leaves of Mhb1-overexpressing plants after application of 5mM active SNP. The differences indicate that accumulation of non-symbiotic hemoglobin may have a protecting effect against NO-related damages.

We have injected the bacteria *Pseudomonas syringae* into adult transformant and control plant leaves to analyze the extent of damage resulting from hypersensitive necrotisation. The hypersensitive response (HR) was suppressed in transformed plant leaves as compared to control leaves after inoculation with the bacteria.

Similarly, when transgenic and control plant leaves were infected with Tobacco Necrosis Virus (TNV), transgenic tobacco lines showed a significant suppression of the lesion number in comparison with control.

ROS and SA levels in untreated and *Pseudomonas syringae*- infected transformant plants were higher in comparison with SR1.

Conclusions

- Mhb1 is the first nonsymbiotic hemoglobin isolated from alfalfa
- *Mhb1* expression in root: may be induced by relative hypoxia
- *Mhb1* gene is induced by hypoxia in suspension cells
- Mhb1 protein level did not change by hypoxia in suspension cells: possible reasons are higher Mhb1 protein turnover or
  unstable mRNA– the potential importance of transcriptional regulatory mechanisms
- *Mhb1* can be found in the nuclei of alfalfa cells
  In a synchronised culture *Mhb1* mRNA level is increased in correlation with respiration values (during G2/M and G1 phases) - it reflects higher metabolic activity during specific cell cycle phases and the induction of *Mhb1* gene during higher metabolic activity
- NO retards the growth of Mhb1 overexpressing seedlings to a smaller extent than the growth of nontransformants– this may be the

consequence of an intracellular NO level decrease resulting from interaction between Mhb1 and NO

- NO causes less necrosis on mature leaves of transfomed plants than on SR1 leaves–it is possible that the interaction of Mhb1 and NO results in intracellular NO level decrease
- It is probable that interaction of Mhb1 and NO causes the change of NO–dependent response to pahogens which can be the cause of the smaller necrotized areas on transformed plant leaves
- The higher ROS and SA levels measured in infected leaves of transformed plants can only be a partial compensation for the lack of NO– related signalling mecahnisms

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Publication list

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