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

PhD Theses
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List of Abbreviations

RBS: Roussin's black Salt

SNP: Sodium nitroprusside

HR: Hypersensitive response

NO: Nitric oxide

cGMP: Cyclic guanosine monophosphate

cADPR: Cyclic adenosine diphosphate-ribose

ROI: Reactive oxygen intermediates

ATP: Adenosine triphosphate

NADH: Nicotinamide adenine dinucleotide

PAL: Phenylalanine ammonia-lyase

PR-1: Pathogenesis-related 1 protein

SA: Salicylic acid

MAP: Mitogen-activated protein kinase

NOS: Nitric oxide synthase

IRP: Iron regulatory protein

2,4-D: 2,4 dichlorophenoxyacetic acid

NBT: Nitroblue tetrazolium

Introduction

The discovery of plant non-symbiotic hemoglobins, or "phytoglobins", (Appleby *et al.*, 1983; Bogusz *et al.*, 1988) came significantly later than that of symbiotic hemoglobins (Kubo, 1939). Although the function of the symbiotic hemoglobins had long been known (Bergensen *et al.*, 1973) by that time, of course this has raised many new questions, and by far not all of them have been answered yet. The shortness of time that has passed since then is of course just one reason, and maybe not the most important one. There are new results in other, but related fields, the appearance of which was necessary for asking those questions that can take us closer to the understanding the role of these proteins.

The tight oxygen-binding properties of non-symbiotic hemoglobins were the first signs to show a function which was quite different from what symbiotic hemoglobins were good for (Arredondo-Peter *et al.*, 1998). Inducibility by hypoxia and evidence for barley hemoglobin to take part in ATP metabolism also supported the distinct function of non-symbiotic hemoglobins (Sowa *et al.*, 1998). Another milestone was to discover the importance of NO-binding by hemoglobins in animals (Gow and Stamler, 1998). The investigation of the possible functions of NO in plants has led to new and interesting results recently. These results suggested that many functions of NO in plants are very similar to its function in animals (Hausladen and Stamler, 1998; Wendehenne *et al.*, 2001). Furthermore, it has been shown that not only animal hemoglobins, but also hemoglobins of unicellular organisms can interact with NO in a physiologically significant way (Hausladen *et al.*, 1998; Minning *et al.*, 1999). Only recently it has been reported that NO and phytoglobins could interact with each other during hypoxia (Dordas et al., 2002). In this thesis we summarize the results obtained by the characterization of Mhb1, the first non-symbiotic hemoglobin isolated from alfalfa on molecular (DNA, RNA and protein) and supramolecular (transformant plants overexpressing the Mhb1 protein) levels with

particular respect to its involvement in the NO-signalling pathway. Furthermore, its other possible functions have also been investigated, such as its involvement in the metabolically active processes, *e.g.* cell division.

Chapter 1 – Literature review

1.1. General remarks on hemoglobins

Hemoglobins are common to be found in various organisms ranging from unicellulars to higher plants and animals (Poole, 1994; Gilles-Gonzalez *et al.*, 1994; Wittenberg and Wittenberg, 1990; Appleby, 1992). In unicellular organisms these proteins are bifunctional (Poole, 1994) since they have a globin domain for oxygen-binding and a flavin prosthetic group with NADH oxidizing activity (Iwaasa *et al.*, 1992; Poole *et al.*, 1996). Kinase domain can also be found as their second domain, *e.g.* in the case of the FixL protein of *Rhizobium meliloti* (Gilles-Gonzalez *et al.*, 1994).

The hemoglobins of higher organisms are either monomeric (*e.g.* leghemoglobin, Appleby, 1992) or multimeric proteins, such as mammalian myoglobin and hemoglobin (Wittenberg and Wittenberg, 1990) and some plant non-symbiotic hemoglobins (Hill, 1998). It is well known that animal hemoglobins bind and carry the gases of respiration, mainly O_2 and CO_2, and this is considered to be their main function. Plant hemoglobins can also bind these gases, and they are usually divided into three major groups on the basis of their common features.

1.2. Plant hemoglobins

The first group is formed by symbiotic plant hemoglobins. 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 (Appleby, 1984).

Non-symbiotic plant hemoglobins, termed also as "phytoglobins"(Dordas *et al.*, 2002), belong to the second group. They are not only present in plants containing symbiotic hemoglobins (Appleby, 1984; Andersson *et al.*, 1996; Appleby *et al.*, 1983; Christensen *et al.*,

1991; Landsmann *et al.*, 1986), but also in other plant species such as *Arabidopsis* (Trevaskis *et al.*, 1997), barley (Trevaskis *et al.*, 1994), rice (Arredondo-Peter *et al.*, 1997), *Trema tomentosa* (Bogusz *et al.*, 1988), *etc*. Furthermore, symbiotic hemoglobins are assumed to have evolved from non-symbiotic hemoglobins by gene duplication (Landsmann *et al.*, 1986). That is why non-symbiotic hemoglobins are considered not only to be more widespread, but also more ancestral than symbiotic hemoglobins (Andersson *et al.*, 1996). This gave a basis to call non-symbiotic hemoglobins as phytoglobins, while other plant hemoglobins with specific function and location could be defined with the appropriate adjective or suffix (Dordas *et al.*, 2002). Because the group of non-symbiotic hemoglobins has only been discovered relatively recently (Appleby *et al.*, 1983; Bogusz *et al.*, 1988), their function is still being investigated.

In general, the members of this group have much higher affinity for oxygen compared to symbiotic hemoglobins (Duff *et al.*, 1997; Arredondo-Peter *et al.*, 1998) and are induced in plants under low oxygen tensions, which attributed an oxygen sensing function to phytoglobins (Trevaskis *et al.*, 1997; Taylor *et al.*, 1994). As an exception, a hemoglobin gene (*AHB2*) from *Arabidopsis thaliana* with close homology to non-symbiotic hemoglobins was shown to be induced by cold stress, not by hypoxia (Trevaskis *et al.*, 1997). This gave the basis for the classification of non-symbiotic hemoglobins. According to Trevaskis *et al.*, to class 1 those hemoglobins belong that are induced upon hypoxic treatment. The cold-inducible *AHB2* non-symbiotic hemoglobin gene belongs to class 2 (Trevaskis *et al.*, 1997).

However, it is not only hypoxia where class 1 genes have a role. *E.g.* the class 1 *Ahb1* gene of *Arabidopsis thaliana* could also be induced by nitrate (Wang *et al.*, 2000). Furthermore, 2,4-dinitrophenol, a respiratory chain uncoupler, was shown to increase both oxygen consumption and barley hemoglobin expression in barley aleurone tissue. This indicated that the expression of barley hemoglobin is influenced by the availability of ATP in the tissue. (Nie and Hill, 1997).

Barley non-symbiotic hemogobin was also shown to be involved in ATP metabolism under hypoxia. It was observed that the ATP levels of a maize suspension culture overexpressing barley hemoglobin were about 30% higher than that of nontransformed maize cells, both grown under hypoxic conditions. On the other hand, the ATP content of maize suspension cells containing an antisense barley hemoglobin construct was about 30% less than that of the non-transformed maize cells when both cultures were grown under hypoxia (Sowa *et al.*, 1998).

High mRNA levels of phytoglobin were observed in the roots and rosette leaves of barley (Taylor *et al.*, 1994), young leaves, stems and roots of soybean (Andersson *et al.*, 1996), in rice leaves and roots (Arredondo-Peter *et al.*, 1997) and in *Arabidopsis thaliana* roots (Trevaskis *et al.*, 1997). The accumulation of phytoglobin mRNA under non-hypoxic conditions is believed to occur because of the high metabolic activity of the above mentioned tissues (Andersson *et al.*, 1996).

The third major group involves 2-on-2 plant hemoglobins. Their name implies a structural difference from the other two major groups, and their function is also thought to be different. Although they have some similarity to non-symbiotic hemoglobins, they have unique biochemical properties and evolutionary history. They show the highest homology to the truncated hemoglobins of microorganisms. Such a gene was found in *Arabidopsis thaliana* roots and shoots (*AHB3*), and shown to be down regulated by hypoxia (Watts *et al.*, 2001).

1.3. Transgenic plants overexpressing plant hemoglobins

No full reports have appeared so far about transgenic plants overexpressing non-symbiotic hemoglobin. However, transgenic tobacco expressing bacterial (*Vitreoscilla sp.*)

hemoglobin has already been generated. Plants overexpressing this hemoglobin showed enhanced dry matter and chlorophyll content, faster germination and earlier flowering times than the wild-type control plants. These transgenic plants also contained higher levels of nicotine. These results suggested that plants expressing bacterial hemoglobin had a higher level of cellular oxygenation (Holmberg *et al.*, 1997). This hemoglobin has also caused an increased growth after transforming it to *Escherichia coli* (Khosla and Bailey, 1988). It is important to remark that the dissociation constant of the *Vitreoscilla* hemoglobin is much higher than that of phytoglobins (Bülow *et al.*, 1999), which makes the above changes in growth reasonable.

1.4. Recently discovered roles of hemoglobin

In unicellular organisms and animals it has been demonstrated that most hemoglobin proteins interact with NO in some physiologically important way. The study of this interaction gave then way to the recent discovery that the role of hemoglobin in these organisms is not restricted to the simple molecule-carrier function in respiration. Mammalian hemoglobin interacts with NO either to form S-nitroso- (when NO is bound to cysteine β_{93}) or nitrosylhemoglobin (here NO is bound to heme) in the arterioles. Then, on entering the lung, hemoglobin undergoes an allosteric transition (from T to R conformation) induced by oxygen, during which all the NO groups are transferred from hemes to cysteine β_{93}. This molecule, the S-nitroso-oxyhemoglobin (with NO bound to thiol and O_2 to heme), enters the arterial circuit. When it reaches the arterioles and capilleries again, low oxygen tension induces the allosteric transition back to the T state. At the same time the NO is released from cysteine β_{93}. Since NO has a vasodilatatory effect, it dilates blood vessels and thereby facilitates O_2 delivery (Stamler *et al.*, 1997; Gow and Stamler, 1998; Gow *et al.*, 1999).

However, NO can interact with hemoglobin not only in the mammals. In the nematode, *Ascaris*, hemoglobin is thought to act as a deoxygenase, using NO to detoxify oxygen in this

aerophobic organism (Minning *et al.*, 1999). In bacteria, the flavohemoglobins are thought to act as dioxygenases using O_2 to detoxify NO in order to avoid nitrosative stress (Hausladen *et al.*, 1998).

1.5. Nitric oxide formation in animals and plants

In animal cells, the biosynthesis of NO is primarily catalyzed by different isoforms of the enzyme nitric oxide synthase (NOS) (Nathan and Xie, 1994). NOSs can oxidize L-arginine to L-citrulline and NO. NOS-like activity, based on the formation from L-arginine to L-citrulline or on the sensitivity to mammalian NOS inhibitors has been detected in several plants, but no plant *NOS* gene has been identified yet (Delledonne *et al.*, 1998; Ribeiro *et al.*, 1999). NO is also produced enzymatically from NO_3^- in plants by the NAD(P)H-dependent nitrate reductase. Furthermore, in plants, non-enzymatic NO-formation is also possible as a result of nitrous oxide decomposition and of chemical reaction of nitrite at acidic pH (Wendehenne *et al.*, 2001).

1.6. NO-related effects in plants

Multiple and important effects and functions of NO have been discovered in plants recently. Some of them coincide with NO functions already described in animals. *E.g.* in animals, NO can function as a messenger involved in several pathophysiological processes including programmed cell death (Messmer *et al.*, 1994) and those of immune, nervous and vascular systems (Schmidt and Walter, 1994; Van Camp *et al.*, 1998). The NO signalling in animals can either be cyclic guanosine monophosphate (cGMP)-dependent or- independent (Hausladen and Stamler, 1998). In the cGMP-dependent pathway NO activates guanylate cyclase and the cell's cGMP level is increased. Here cGMP is involved in smooth muscle

relaxation, inhibition of platelet aggregation and in sensory systems (Schmidt and Walter, 1994).

NO has cGMP-independent biological effects in animals in the form of peroxynitrite ($ONOO^-$). Peroxynitrite is formed from NO and the NADPH oxidase product superoxide (O_2^-), and is believed to play a role in apoptosis in animals (Beckman *et al.*, 1994). The situation of peroxynitrite formation is similar in plant defense responses (Wendehenne *et al.*, 2001), but its importance was shown to be less in the hypersensitive reaction (HR) than in animal cells (Delledonne *et al.*, 2001).

Another cGMP-independent reaction is nitrosylation, which can modify signal transduction. There are NO-responsive signalling proteins (receptors, ion channels, enzymes and transcription factors) that either have transition metal prosthetic groups or thiol/tyrosine residues where NO can exert its effect (Stamler *et al.*, 1992; Stamler, 1994). One such protein that NO activates by S-nitrosylation is $p21^{ras}$ (Stamler et al, 1992). This leads to the induction of MAP kinase cascades which can induce apoptosis (Lander *et al.*, 1996). NO has been shown to indirectly modify the MAP kinase activity in mammalian tumor cells and neurons (Mott *et al.*, 1997; Yun *et al.*, 1998). It is important to remark that NO can also inhibit apoptosis. (Kim *et al.*, 2001).

Recent evidence suggests that NO-induced cGMP synthesis is required for NO-induced cell death of cultured *Arabidopsis* cells through the activation of a MAP-kinase upon incubation with different concentrations of NO-donor compounds: sodium nitroprusside (SNP) or Roussin's black salt (RBS). (Clarke *et al.*, 2000). Apoptotic cell death was also shown to be induced by NO in *Taxus* callus cultures (Pedroso *et al.*, 2000).

In plants, the cGMP levels showed a transient increase not only upon addition of NO, but also following gibberelic acid and light stimulation in barley aleurone, bean cells and *Pinus*

needles (Wendehenne *et al.*, 2001). Similarly, the cGMP levels of tobacco increased when cells were treated with NO (Durner *et al.*, 1998).

NO, similarly to its activatory role in mammalian defense responses (Schmidt and Walter, 1994; Stamler, 1994), is an important component of the plant disease resistance system (Delledonne *et al.*, 1998). Application of NO donor compounds to or overexpression of recombinant mammalian NOS in tobacco plants or cell suspensions induced the expression of the defence genes encoding the pathogenesis-related-1 (PR-1) protein and the enzyme phenylalanine-ammonia lyase (PAL). Furthermore, these genes proved to be inducible by cGMP-analogues, too. The induction of PR-1 and PAL was also observed partly as a consequence of the increasing cyclic adenosine diphosphate-ribose (cADPR) levels either directly (through S-nitrosylation) or indirectly (in a cGMP-dependent way) induced by NO (Figure 1). Consequently, cGMP and cADPR are second messengers of NO in plants, and they can act synergistically (Klessig *et al.*, 2000), just as reported for gene activation in animal cells (McDonald and Murad, 1995).

Hypersensitive reaction (HR) means the process of necrotic lesion formation at the site of pathogen entry in order to prevent the pathogen from spreading to uninfected tissues. The first step of the plant hypersensitive reaction is an oxidative burst when so called reactive oxygen species (ROS) like superoxide radical (O_2^-) and hydrogen peroxide (H_2O_2) are generated upon pathogen infection. This burst leads to several effects including the cross-linking of the cell wall (Bradley *et al.*, 1992) and the induction of various plant genes (such as glutathione S-transferase and glutathione peroxidase) involved in cellular protection and defence including the above mentioned PR-1 and PAL (Figure 1, Levine *et al.*, 1994; Jabs *et al.*, 1997). Salicylic acid (SA) is a signalling molecule that contributes to H_2O_2 production during HR, but high ROS levels can also stimulate SA-production (Chamnongpol *et al.*, 1998). Sometimes NO was observed to act synergistically with SA and ROS (Stamler, 1994; Klessig *et*

al., 2000). In soybean, a huge oxidative burst upon pathogen infection could cause only a weak induction of cell death, but the addition of NO significantly increased the strength of the response (Delledonne *et al.*, 1998). The relationship between NO, SA and ROS has been described recently as a self-amplifying process during which redox signalling through NO and ROS can be enhanced by salicylic acid (Van Camp *et al.*, 1998). However, NO can also act independently of ROS to induce the expression of defence-related genes in the case of *Arabidopsis* cell suspension culture (Clarke *et al.*, 2000) and tobacco (Durner *et al.*, 1998). The relationship of other signalling molecules (*e.g.* ethylene, jasmonic acid) in HR to the above mentioned ones is still being studied intensively.

Furthermore, the oxidative burst has a direct effect leading to host cell death through the Fenton reaction which means the formation of highly reactive species from the less reactive ones (Lamb and Dixon, 1997; Levine *et al.*, 1994). Fenton (or Haber-Weiss) reaction occurs in the presence of free iron in the cytoplasm. The more free iron is present, the more lethal the reaction is. The mRNA binding protein IRP-1 is known to increase intracellular free iron levels in animals by binding to ferritin mRNA and preventing it from translation. IRP-1 is generated from the enzyme aconitase, the activity of which is previously inhibited by binding NO (Hentze and Kühn, 1996). Plant aconitases have high homologies to human IRP-1 protein, and their activities were also inhibited by NO, suggesting contribution to the defence mechanism against pathogens (Figure 1, Navarre *et al.*, 2000).

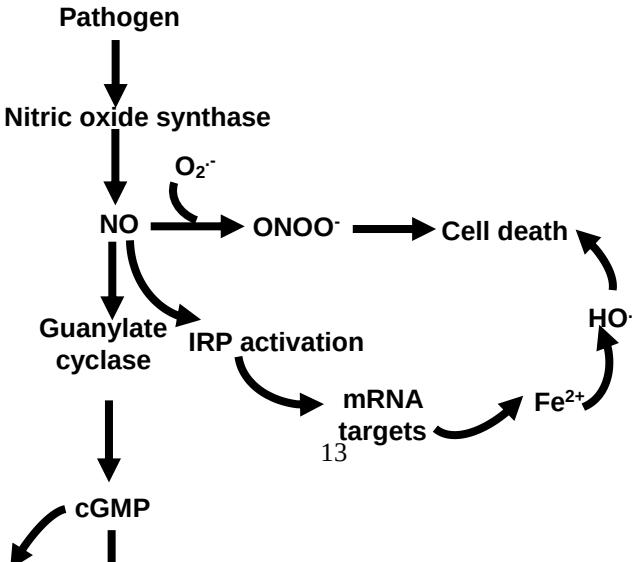

Figure 1

NO-mediated signalling pathway in plant defense against pathogens (Wendehenne *et al.*, 2001)

1.7. Further effects of NO in plants

During hypoxia NO production was observed in maize cell cultures and alfalfa root cultures. Similarly to the earlier described situation in bacteria, phytoglobins could help detoxify this compound (Figure 2). This hypothesis 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 (Dordas *et al.*, 2002).

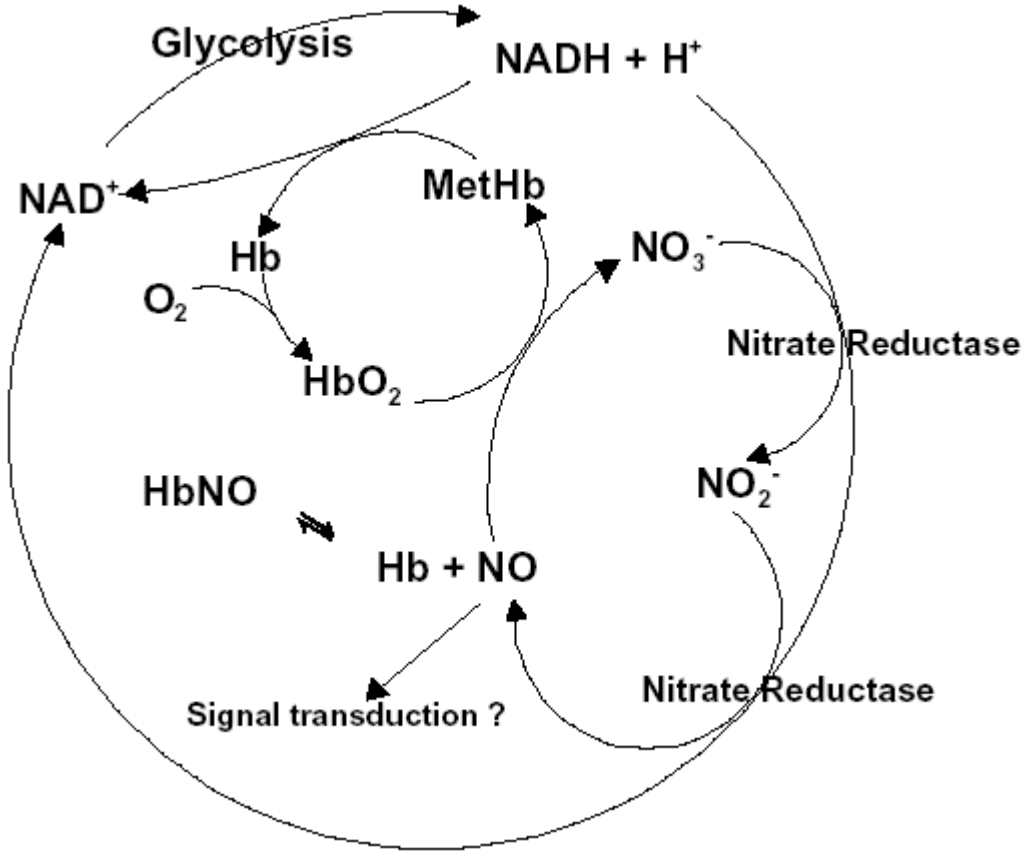

Figure 2

Summary of the reactions involving both NO and phytoglobin in plant cells during hypoxia (Dordas et al., 2002)

As for the interaction of NO with leghemoglobins, there have been suggestions that the reaction of NO, formed from NO_3^-, with leghemoglobin may be a possible mechanism by which nitrate inhibits nitrogen fixation in legume nodules (Kanayama and Yamamoto, 1991).

Although it seems that the major role of NO is in plant defence mechanisms, it could also evoke light responses such as stimulation of seed germination, de-etiolation and inhibition of hypocotyl elongation on lettuce and *Arabidopsis thaliana* seedlings (Beligni and Lamattina, 2000). It was found to play a part in the control of root growth (Ribeiro *et al.*, 1999) and in plant endogenous maturation and senescence (Leshem *et al.*, 1998), as well.

Since some key effects of NO are mediated by interactions with hemoglobin in very distantly related organisms, it was speculated that plant hemoglobins could also have a similar role (Durner *et al.*, 1999).

In this PhD thesis the characterization of Mhb1, a novel phytoglobin isolated from alfalfa is described with respect to its possible physiological functions including its role in the NO signalling pathway.

Chapter 2 – Aims of Study

Since little has been known about phytoglobins, my purpose 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?

Chapter 3 – Materials and methods

In this chapter all the methods used in the „Results" section (Chapter 4) are listed. Those that differ from traditionally used protocols are described in a more detailed way, while description of the other methods contain sufficient information including references to be able to follow the experiments.

3.*1. Plant material*

A highly homogenous, fast-growing cell suspension culture of *Medicago sativa* ssp. *varia* (genotype A2, tetraploid) was established and maintained at 22-24 ^{o}C in Murashige and Skoog medium (Murashige and Skoog, 1962) in the presence of 1 mg/l 2,4-D and 0.2 mg/l kinetin (Bögre *et al.*, 1988).

Seeds of nontransformed tobacco (*Nicotiana tabacum* cv. Petit Havanna line SR1) and seeds from the third generation of the *Mhb1*-transformant tobacco plants were sown in soil and grown under normal greenhouse conditions (18-23^{o}C; supplementary light: 160 μE $m^{-2}s^{-1}$ for 8h/day; relative humidity: 75-80%).

Medicago sativa ssp. *varia* plants were grown under normal greenhouse conditions as described above.

3.2. Construction and random sequencing of an alfalfa cDNA library

An alfalfa cell suspension cDNA library was constructed with a cDNA Library Construction Kit purchased from Clontech (Palo Alto, CA, USA) according to the instructions of the manufacturer. On random clones from this cDNA library automated sequencing was performed on an ABI DNA sequencer model 373 (Applied Biosystem, Foster City, CA, USA).

3.3. Genomic hybridization

Southern hybridization of the *Mhb1* gene was carried out on filters containing *Dra*I digested genomic DNA of diploid *Medicago sativa* plants. These individuals belong to a segregating F2 population of self-mated progeny of F1/1 which originated from the cross between *Medicago sativa* ssp. *coerulea* (male parent) and *Medicago sativa* ssp. *quasifalcata* (female parent) used earlier for genetic mapping by Kaló *et al.* Conditions for hybridization were the same as described by Kaló *et al.*(2000).

3.4. Synchronization of the cell cycle and flow cytometric analysis

Alfalfa suspension cells were treated with hydroxyurea (Sigma, St. Louis, MI, USA) at a concentration of 10 mM for 36 hours to arrest cell division cycle. Then the cells were washed three times with fresh Murashige and Skoog medium, and cultured further for synchronous growth (Magyar *et al.*, 1993). The isolation of nuclei and the flow cytometric analysis (on a FacScalibur flow cytometer, Becton-Dickinson, USA) were performed according to Savouré *et al.* (Savouré *et al.*, 1995).

3.5. Stress treatments

Hypoxia treatment: Gaseous N_2 was bubbled for 90 s through 50 ml of alfalfa cell suspension culture in a 100 ml culture flask which was then closed in an airtight manner, and was left unshaken for 24 hours. Samples were taken at 2 h, 6 h, 12 h and 24 h. N_2 bubbling for 90 s through the sample was repeated after each sample taking step. The samples were snap-frozen in liquid nitrogen and stored at -70 ^{o}C.

Cold stress treatment: A culture flask containing alfalfa cell suspension was placed to 4 ^{o}C and was shaken there for 24 hours. Samples were taken at 0 h and 24 h, they were frozen in liquid nitrogen and stored at -70 ^{o}C.

3.6. RNA isolation and Northern analysis

Total RNA was prepared from frozen alfalfa cells harvested at the indicated timepoints, according to the procedure described by Soni and Murray (1994) with a slight modification to scale the method to the volume of Eppendorf tubes. Total RNA was quantified on the basis of optical density at 260 nm. Twenty micrograms of total RNA was loaded on 1% formaldehyde agarose gel containing 0.01% ethidium bromide. Transferring RNAs to Hybond N^+ filter (Amersham, England) was performed with the capillary action technique, and the filter was examined under UV light to verify the efficiency and the quality of transfer. Hybridization of the filter was performed in hybridization buffer containing 5mM ethylenediaminetetraacetic acid (EDTA), 50 mM pH 7 mixture of Na_2HPO_4 and NaH_2PO_4, 900 mM NaCl, 100 μg/ml tRNA, 1x Denhardt's reagent and 0.1% sodium-dodecyl-sulphate at 65 ^oC (Sambrook *et al.*, 1989). Radiolabelled probes were generated by random-primed ^{32}P-labelling of the coding regions of *CycIIIMs* (Savouré *et al.*,1995) and *Mhb1* and cDNA fragments corresponding to the 3' nontranslated regions of the actin gene from *Medicago truncatula* (GenBank accession number: AA660319) and *Mhb1*.

3.7. Respiration rate determination

Three times 1 ml cell suspension aliquots were taken from the synchronously growing culture right after removing hydroxyurea and every two hours thereafter, and were immediately placed in an airtight oxygen electrode chamber (Hansatech oxygen electrode unit DW2/2, Clark type). Data were measured by Hansatech O_2 control box type CB1-D.

The respiration rate was determined by dividing the measured oxygen consumption values by the fresh cell weight of all three aliquots, respectively. From the three respiration rate values we calculated a mean value, and this was plotted together with the standard

deviation values. To follow the changes in respiration rate more easily, the mean respiration rates were expressed as percentages of the mean respiration rate of an actively growing unsynchronized culture, which originally was 4.38 μM O_2/hour/g, and was considered as 100%.

3.8. Protein extraction, antibody raising, immunoblotting

Proteins were extracted by grinding frozen alfalfa cells harvested at the indicated timepoints with quartz sand in homogenization buffer containing 25 mM Tris-HCl pH7.7, 75 mM NaCl, 15 mM $MgCl_2$, 15 mM ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), 1 mM dithiothreitol, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride (PMSF), leupeptin and chymostatin, 10 μg/ml each.

Mhb1 hemoglobin gene was first cloned into pTRC HisB expression vector (Invitrogen, Carlsbad, USA). Polyhistidine-tagged Mhb1 recombinant protein was expressed in *E. coli* XL-I Blue cells (Stratagene, USA) and purified with the help of Xpress System according to the instructions of the manufacturer (Invitrogen, Carlsbad, USA). Polyclonal antibody against recombinant Mhb1 protein was raised in mice.

For immunoblotting, the SDS-polyacrylamide gels loaded with 50 μg of protein were transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA) in 50 mM Tris-base-50 mM boric acid buffer at a constant 10 V overnight. The filters were blocked in 5% milk powder-0.02% Tween 20 in TBS (25mM Tris-Cl pH 7.4, 150 mM NaCl) buffer for 2h at room temperature, reacted with the first antibody in the blocking buffer for 2 h at room temperature, washed three times with TBST (0.02% Tween 20 in TBS pH 7.4) and reacted with horse radish peroxidase-conjugated goat anti-rabbit IgG using the dilution recommended by the manufacturer (Sigma, St. Louis, MI, USA). After washing the membrane three times with TBST, signals were developed using SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA) and detected on X-ray films.

3.9. Immunocytochemical localization of the Mhb1 protein

Alfalfa suspension cells were pelleted by centrifugation and fixed with 1% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.2) for 1 hr. After rinsing in the same buffer, the samples were dehydrated with ethanol and embedded in Lowicryl K4M resin (TAAB Laboratories Equipment Ltd., Berkshire, U.K.). Immunocytochemical localization of the Mhb1 protein was carried out according to Mustárdy *et al.* (1996) ona TESLA BS540 electron microscope.

3.10. Plant transformation

The Mhb1 full length cDNA was cloned into the pRok2 plant expression vector (kindly provided by Anthony Kavanagh, Trinity College, Dublin, Ireland) where its expression is regulated by the Cauliflower Mosaic Virus (CaMV) 35S promoter (Benfey *et al.*, 1989). The plasmid construct was introduced into *Agrobacterium tumefaciens* EHA105 (kindly provided by MOGEN, Leiden, The Netherlands) by three-parental mating. Tobacco plants (*Nicotiana tabacum* cv. Petit Havanna line SR1) were infected and co-cultivated with the *Agrobacterium* suspension, and kanamycin-resistant plants were regenerated according to the method described by Horsch *et al.* (1985). Expression of the *Mhb1* gene in tobacco plants was analysed by immunoblotting.

3.11. Nitric oxide treatment of germinating seeds

Seeds derived from the third generation of the *Mhb1*-transformed and nontransformant SR1 plants were put on filter paper moisturised with 3 ml of water and placed to 23 ^{o}C in the light for 48 h. Then 1 ml of SNP was added to the Petri dishes from a 1.2 mM freshly prepared stock solution, so the final SNP concentration was approximately 300 μM. To control seeds the

same volume of water was supplied without SNP. Light-inactivated SNP was prepared from a 1.2 mM SNP stock solution exposed to direct sunlight for 10-12 hours or to artificial light for 2-3 days. The obtained inactive SNP was then used similarly to the above described use of active SNP. Eight-day-old seedlings (on the sixth day of the treatment) were photographed with an Olympus Camedia C2020Z digital camera (Olympus Optical Co. Ltd. Tokyo, Japan) attached to an Olympus (Olympus Optical Co. Ltd. Tokyo, Japan) stereomicroscope. The experiment was repeated three times.

3.12. Treatment of tobacco leaves with nitric oxide, bacteria or virus

For each experiment, 50-60-day-old plants and the third and fourth true leaves (i.e. the third and fourth leaf position above hypocotyl) were used for treatment or inoculation with pathogen.

To study the effect of NO on leaf tissues, 5 mM SNP solutions were injected into the interveinal areas of leaves of control (SR1) and transformed (1, 11, 13) tobacco lines. Light-inactivated and active SNP solutions were injected (infiltrated) with hypodermic syringe and needle into an about 1 cm^2 leaf area. The size of the necrotized leaf area was evaluated periodically after injection. Data are an average of a representative experiment from 4 independent experiments with at least 4 replicates showing similar results.

Pseudomonas syringae pv. *phaseolicola* was maintained on nutrient agar and stored at 4^{o}C. Bacteria for inoculation were cultured on King's B medium (King *et al.*, 1954) at 25^{o}C for 24 h, collected in sterile distilled water (SDW) and centrifuged at 1500 *g* for 10 min. The bacterial pellet was resuspended in SDW and the concentration adjusted to $3x10^8$ ml^{-1} bacteria. The 3rd and 4th tobacco leaves were inoculated using a hypodermic syringe and needle. The development of the hypersensitive reaction was evaluated periodically after inoculation. Data are an average of a representative experiment from 4 independent experiments with at least 4

replicates showing similar results.

The 3rd and 4th leaves of control and transformed tobacco plants were inoculated with a suspension of Tobacco Necrosis Virus (TNV). Leaves of *Nicotiana tabacum*. cv. Samsun plants showing typical disease symptoms of TNV were ground in a mortar (1g in 3 ml 10 mM Na-phosphate buffer, pH 7.0), and this was used for inoculation of tobacco leaves. Data are an average of a representative experiment from 3 independent experiments with at least 4 replicates showing similar results.

3.13. Measurement of ROS production

ROS concentrations were measured on 10 leaf discs obtained from fully developed leaves of healthy plants or in different timepoints after pathogen treatment. ROS production was assayed spectrofotometrically according to Doke's method (1983) by monitoring the reduction of nitroblue tetrazolium (NBT from SIGMA) at 580 nm after incubation with leaf discs isolated from appropriate plants.

3.14. Quantitation of SA

Two month old plants were injected with *P. syringae* pv. maculicola at a concentration of 10^8 cfu/ cm^3. Inoculations were performed on 3 plants during each experiment. Free SA and conjugated SA (SAG) levels were determined from the same leaf material at each timepoint.

Free SA and SAG were extracted and quantitated essentially as described by Hennig *et al.* (1993) and Malamy *et al.* (1992). HPLC was performed as described (Malamy *et al.,* 1990) on a C-18 reverse phase column (Macher&Nagel) using HPLC system and Fluorescence Detector from Shimadzu, with the excitation wavelength of 305 nm and emission wavelength of 410 nm.

Köszönetnyilvánítás

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Végül, de nem utolsósorban köszönöm szüleim minden szeretetét, segítségét és támogatását.

Chapter 4 – Results

4.1. A non-symbiotic hemoglobin gene from alfalfa

In sequenced alfalfa cDNAs we have found a 483 basepair long gene that we identified with the help of the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST) as *Mhb1*, a 160 amino acid long (18 kD) non-symbiotic non-leghemoglobin. It turned out from sequence alignments (Figure 3) that *Mhb1* is a full length cDNA and has all the features that are characteristic of plant hemoglobins. These features include strong homology to other non-symbiotic hemoglobins and conserved amino acid residues (Ota *et al.*, 1997). Plant globin motif is also present in the amino acid sequence of Mhb1 protein (PROSITE search, http://genomic.sanger.ac.uk/pss/pss.shtml), but not exactly in the [SN]-P-x-L-x(2)-H-A-x(3)-F form because in the case of Mhb1 protein the proline in the motif is substituted by threonine. PROSITE search has also revealed a cAMP- and cGMP-dependent protein kinase phosphorylation site ([RK] (2)-x-[ST]), amino acids from position 26 to 29).

4.2. Genomic analysis and mapping of the Mhb1 gene

To determine *Mhb1* gene copy number and to investigate if there are any cross-hybridizations with leghemoglobins or to other types of non-symbiotic hemoglobins, Southern hybridization was performed with genomic DNA isolated from diploid *Medicago sativa* plants and cDNA probe from *Mhb1* gene. The hybridization resulted in three strong bands on the *Dra*I-digested genomic DNA of diploid alfalfa plants (Figure 4) which could indicate the presence of *Mhb1* gene either in single copy or low copy number.

```
MHB1   MGTLDTKGFTEEQEALVVKSWNAMKKNSAELGLKLFLKIFEIAPSAQKLFSFLKDSKVPL 60
CASNON .S..EGR.............S...P.AG.....F.....................N... 60
```

```
SOYNON .T.TLER..S...........V.....G.....F.................R..T... 60
TREMA        .V...........AV..........F...........KN...Y....PI.. 54
PARA         .V..........A.AV.........QF...........KN...Y....P... 54
AHB1           ............SV............I.......TTK.M....R..PI.A 52
RICE2          .S.......L...AI...D..NI..RF......V....SQM....RN.D... 52
BARLEY         .S..K....L...AI...D..N...RF...........RQM.P..R..D... 52
RICE1          .S.......L...AIL..D..NIA.RF......V....SQM....RN.D... 52
AHB2           ....K.....KE..EIL.QDIPKYS.HF.SQ.L....A.KG.....R..DEVP 53
CASSYM           ..K....LKQ..EVL.Q.IPAHS.R..AL.I.A..ESKYV.......NEIP 51
MLB1           ..DK.....NS..E.F.Q.LPRYSVFFYTVVL.K..A.KG......N.-AEV 51
PEALB          ....K.....NS..ELF.Q.PS-YSVLFYTI.LKK..A.KGM.......AEVV 52

MHB1   EQNTKLKPHAMSVFLMTCESAVQLRKSGKVTVRESSLKKLGANHFKYGVVDEHFEVTKFA 120
CASNON .R.P...S.................A.............S...H..A......... 120
SOYNON ...P......V...V...D.......A........N......T..RT..AN......... 120
TREMA  ...P.......T..V...........A........N..R...I...N...N......R.. 114
PARA   ...P......TT..V...........A.....K..D..RI..I...T...N......R.. 114
AHB1   ...P..........V.C.........T.......TT..R...S.S...........A.Y. 112
RICE2  .K.P...T......V....A.A....A......DTT..R...T......G.A.....R.. 112
BARLEY .T.P...T..V...V....A.A....A..I....TT..R..GT.L....A.G.....R.. 112
RICE1  .K.P...T......V....A.A....A......DTT..R...T.L....G.A....V... 112
AHB2   HN.P...A..VK..K....T.I...EE...V.ADTT.QY..SI.L.S..I.P....V.E. 113
CASSYM .N.P...A..AVI.KTI....TE..QK.HAVWDNNT..R..SI.L.NKIT.P....M.G. 111
MLB1   QDSPQ.QA..EK..GLVRD......AT.G.VLGDAT.---..I.VRK....P..V.V.E. 108
PEALB  D-SP..QA..EK..G.VHD..I...A..E.VLGDVT.---..I.IQK..I.P..V.V.E. 108

MHB1   LLETIKEAVP-EMWSPAMKNAWGEAYDQLVNAIKSEMKPSS 160
CASNON ..........-.T...E.........K..A...L...... 160 (86%)
SOYNON ..........-...................D........P. 160 (85%)
TREMA  ..........-.....E.............A.......... 154 (85%)
PARA   ..........-.....E......V......A...F...... 154 (82%)
AHB1   ..........-.....E..V...Q...H..A...A..NL.N 152 (79%)
RICE2  ..........VD.......S..S...N...A...Q....A  153 (74%)
BARLEY ........L.AD..G.E.R...........A...Q....A  153 (72%)
RICE1  ..D....E..AD.......S..S....H..A...Q....A  153 (71%)
AHB2   ..R.L..GLG-.KYNEEVEG..SQ...H.AL...T...    150 (52%)
CASSYM ..G.....IK-.N..DE.GQ..T...N...AT..A...    148 (51%)
MLB1   ..K.....AG-DK..EELNT..EV...A.AT...KA.     144 (45%)
PEALB  ..D.....SG-.K..EELST..EI..EG.AS...KA.     144 (46%)
```

Figure 3

Alignment of some plant hemoglobin protein sequences using the BLASTP program

The highly conserved residues of heme and ligand binding (the distal and proximal histidines, phenylalanine CD1 and proline CD2) are in bold (Ota *et al.*, 1997). Plant globin motif characteristic of plant hemoglobins is shown underlined. Dots mean identical amino acids. Percentages in brackets show values of amino acid identity to Mhb1 protein. Hemoglobins (with GenBank accession numbers or with references): MHB1: this study (AF172172), CASNON: *Casuarina glauca* non-symbiotic hemoglobin (X53950), SOYNON: soybean non-symbiotic hemoglobin (U47143), TREMA: *Trema tomentosa* non-symbiotic hemoglobin (Y00296), PARA: *Parasponia andersonii* hemoglobin (U27194), AHB1: *Arabidopsis thaliana* class1 non-symbiotic hemoglobin (U94998), RICE2: rice class 2 non-symbiotic hemoglobin (U76028), BARLEY: barley non-symbiotic hemoglobin (U01228), RICE1: rice class 1 non-symbiotic hemoglobin (U76029), AHB2: *Arabidopsis thaliana* class 2 non-symbiotic hemoglobin (U94999), CASSYM: *Casuarina glauca* symbiotic hemoglobin (Kortt *et al.*, 1988), MLB1: *Medicago sativa* class 1 leghemoglobin (X13375), PEALB: pea leghemoglobin (AB015720).

Since the DNA was isolated from individuals of diploid *Medicago sativa* F2 segregating population, it was possible to determine by following their inheritance that all hybridization bands represent the alleles of a single gene. The same population had been used earlier to construct the detailed genetic map of alfalfa (Kaló *et al.*, 2000), so with the help of the already existing genotype data (based on segregation patterns) the genetic mapping *Mhb1* was also carried out. This gene located at the 40-47 cM region of linkage group 4 between two codominant RAPD markers, OPA12A and OPA17A. We compared the *Mhb1* hybridization pattern to that of previously used leghemoglobin gene probes, but no cross-hybridization between leghemoglobins and the coding region of *Mhb1* gene could be detected. However, as can be seen on Figure 4, besides the strong hybridization signals of the alleles of the single *Mhb1* gene, weakly cross- hybridizing bands can also be observed. These weak signals possibly could appear as a consequence of *Mhb1* coding region hybridizing to other, still unidentified types of non-symbiotic hemoglobins in alfalfa.

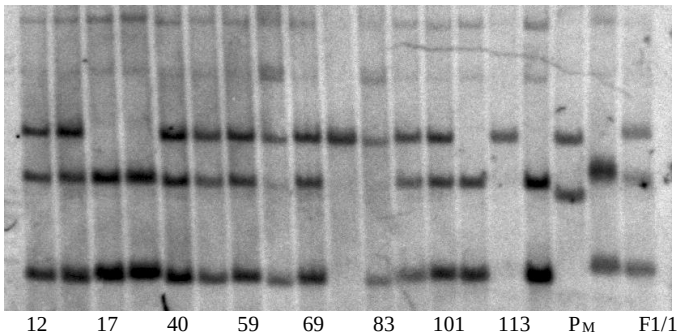


Figure 4

DNA-DNA hybridization analysis and genetic mapping of the alfalfa non-symbiotic hemoglobin gene *Mhb1*

P_M and P_F are male and female parental plants, respectively. Numbers identify the individuals of self-mated progeny of the F1/1 plant. Since the numbered individuals were earlier used to construct the genetic map of alfalfa (Kaló *et al.*, 2000), on the basis of segregation patterns it was also possible to deteremine the linkage properties of the *Mhb1* gene.

4.3. Expression of Mhb1 in various alfalfa organs

We examined the tissue specificty of the *Mhb1* gene expression in alfalfa. Northern analyses of total RNA samples obtained from various plant tissues grown under normal greenhouse conditions have revealed detectable *Mhb1* mRNA level only in the roots of alfalfa (Figure 5). Hybridization of the same filter with actin (control cDNA) gene indicated the equal RNA quantity in each lane (Figure 5).

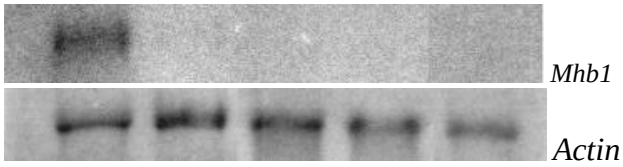


Figure 5

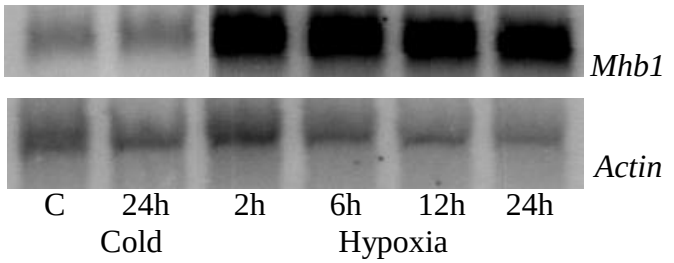
Transcription pattern of the *Mhb1* gene in various plant tissues

The blot was probed with an actin clone from *Medicago truncatula* to verify the equal loading of RNA samples (lower lane).

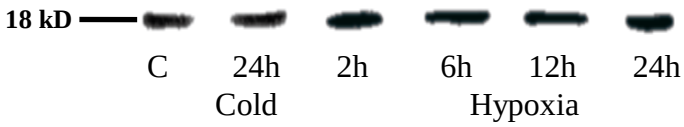
4.4. Induction of Mhb1 gene by hypoxia in cultured alfalfa cells

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 (Figure 6A). In the same culture a significant change in the *Mhb1* RNA amount was detected as a result of the hypoxic treatment. It is clearly visible that the increase in the *Mhb1* transcript level appears as early as 2 hours after the start of hypoxia, and this high level is maintained throughout the experiment (Figure 6A). We hybridized the same RNA filter with actin (control cDNA) gene to show the equal RNA quantity in each lane.

A)



B)



C)

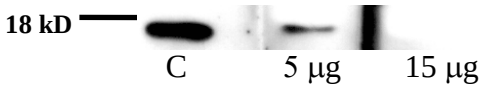


Figure 6

The effect of cold stress and hypoxia on *Mhb1* transcript level and on Mhb1 protein production

(A) Time course analysis of *Mhb1* gene induction under cold stress and hypoxia in alfalfa suspension cells using Northern hybridization. An increase in *Mhb1* mRNA level can be observed under hypoxic conditions only. "C" stands for control. The blot was probed with an actin clone from *Medicago truncatula* to verify the equal loading of RNA samples (lower lane).

(B) Time course analysis of Mhb1 protein accumulation on a Western blot using polyclonal antibody raised against recombinant Mhb1 protein. "C" stands for control.

(C) Depletion of anti-Mhb1 antiserum with increasing amounts of recombinant Mhb1 protein (5 and 15 µg, respectively).

After exposing cultured cells to cold stress and hypoxia, respectively, the Mhb1 protein accumulation was the same as in cells grown under normal conditions (Figure 6B). Before completing the above immunoblot, the anti-Mhb1 antiserum had been checked on a blot of A2 cell suspension total protein extract. The antiserum recognized a single 18 kD band corresponding to Mhb1 protein. To test the specificity of this polyclonal antibody, its aliquots were incubated on ice for one hour with increasing amounts (5 and 15 µg) of purified

recombinant polyhistidine-tagged Mhb1 hemoglobin. This resulted in the gradual disappearance of the signal. (Figure 6C).

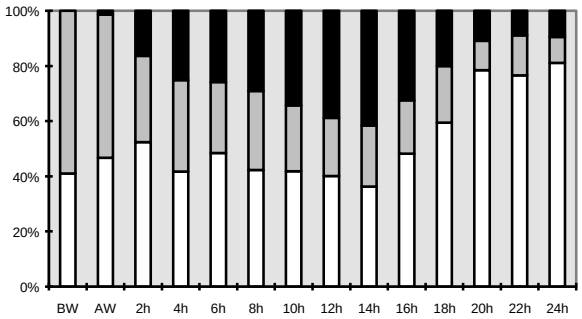
4.5. Cell division cycle- dependent expression of the Mhb1 gene

To test if there is a cell cycle dependence of *Mhb1* gene transcription, we assayed its expression in alfalfa suspension culture synchronized with hydroxyurea. Flow cytometric analysis (Figure 7A) shows that the majority of cells is synchronized in S phase at the beginning of the experiment. Figure 7B shows changes in the *Mhb1* mRNA level during cell division. Although the initial high transcript levels of the *Mhb1* gene could well have been caused by the 36 hour long hydroxyurea treatment, the later RNA level changes at 10-12 hours show the induction of the *Mhb1* gene before mitosis. Control Northern hybridization was carried out with the mitotic cyclin *CycIIIMs* (Savouré *et al.*, 1995) in order to test the quality of synchronization and to follow the progress of cells through mitosis. This shows that G2/M begins at about 12-14 hours (Figure 7C). The relative amount of total RNA in the lanes on the membrane can be seen on Figure 7D.

4.6. Changes in the respiration rate of a synchronized cell suspension culture

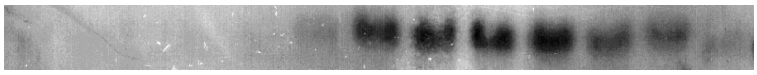
We also wanted to know if there was any detectable change in the metabolic activity of the cells during cell division because it is supposed that there is a correlation between *Mhb1* expression and metabolic activity of the cells (Andersson *et al.*, 1996). That is why respiration rate measurements on the above mentioned synchronized alfalfa cell suspension culture have been carried out.

A)



B)

C)



D)

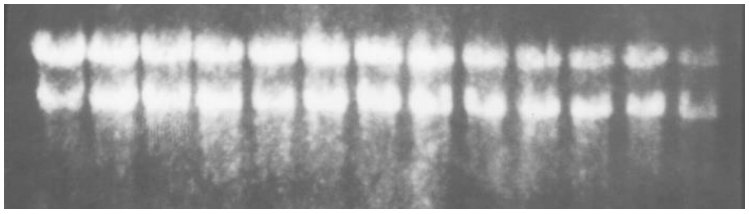


Figure 7

Cell division cycle- dependence of *Mhb1* transcript level in alfalfa suspension culture synchronized with hydroxiurea

(A) Data from flow cytometric analysis show the proportion of cells found in different phases (G2, S, G1, black, grey and white bars, respectively) of the cell division cycle at the examined points of time. "BW" and "AW" stand for "Before Wash" and "After Wash", respectively.
(B) Time course analysis of changes in *Mhb1* transcript levels during cell division cycle of a synchronized alfalfa cell suspension culture (Northern hybridization).
(C) Changes in the mRNA levels of the mitotic cyclin *CycIIIMs* (Savouré *et al.*, 1995) at the examined timepoints of the experiment show that mitosis begins 12-14 hours and ends. 22-24 hours after removing hydroxyurea.
(D) Photograph of the membrane used in the above hybridizations is shown as the control of equal loading.

Since the respiration rate of an actively dividing unsynchronized culture is considered as 100%, Figure 8 shows that the synchronized cells regained their respiratory activity characteristic of normal growth 4 hours after removing hydroxyurea. Furthermore, no change was observable in the respiration rate of the same culture 2 hours after adding hydroxyurea (data not shown). However, in comparison with an unsynchronized culture, there was a decrease in the respiration rate by about 40% measured right before removing hydoxyurea (data not shown). This means that this initial respiration rate change was not the consequence of hydroxyurea itself as a chemical compound, but of its inhibitory effect on cell cycle progression.

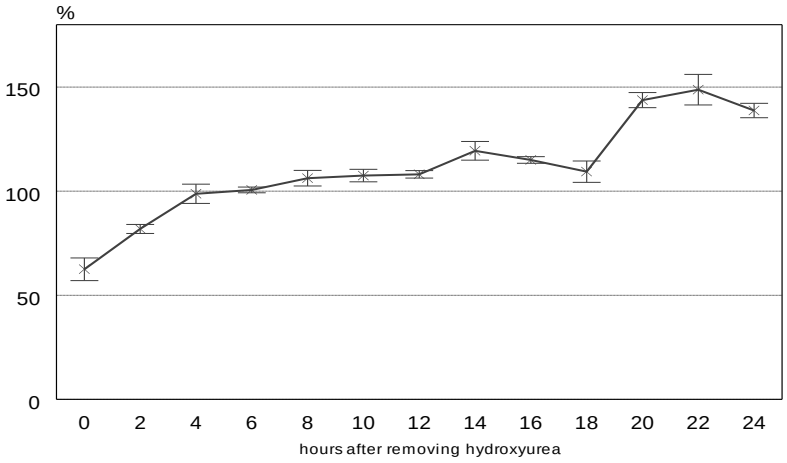


Figure 8

Time course analysis of respiration rate changes of the synchronized alfalfa cell suspension culture.

Respiration rates, expressed as percentages of the mean respiration rate of an actively growing unsynchronized culture, are shown as points marked on the curve. The original 0 hour value is 2.74 μM O_2/hour/g.

Three major changes of the respiration rate can be observed during the cell cycle. At 14 hours there is a reproducibly detectable peak in the respiration rate (from 100% to 119.4%, Figure 8). Between 16 and 18 hours there is a decrease in respiration rate (from 119.4% to 109.4%, Figure 8). From 20 hours after removing hydroxyurea, the respiration

rate strarts to increase again until it reaches 149%, and it remains relatively high (Figure 8).

We should remark that respiration rate values of the alfalfa cell suspension culture synchronized by hydroxyurea may not be accepted as values characteristic at each phase of the cell division cycle. This is because hydroxyurea blocks cell division at G1/S border until its removal. Since the proliferation of cells is blocked at G1/S border, they reduce their overall metabolic activity including respiration rate to a "resting state" value. According to our data, the respiration rate is decreased by about 40% in comparison to that of an unsynchronized culture. After removing hydroxyurea cells are allowed to continue their cell division cycle in a synchronous manner, but from a lower point of metabolic activity than that of untreated cells at G1/S border. The recovery period to reach average respiration value was found to be 4 hours. This means that respiration rate values at least in S phase, cannot be considered as values characteristic for the normal physiological state. However, this should not affect the validity of the later measured values.

4.7. Immunogold localization of the Mhb1 protein

In order to determine the intracellular distribution of Mhb1 protein in alfalfa cells, immuno- electronmicroscopy was carried out. Figure 9A shows the results obtained from immunolabelling with polyclonal antibody raised against recombinant Mhb1 protein. The vast majority of the labelling can be seen within the nucleus, but no label found in the nucleolus. However, to a lower extent, cytoplasmic labelling is also observable. In the control picture, where pre-immune mouse serum was used instead of anti-Mhb1 antibody, no immunogold labelling is visible (Figure 9B). The immunogold localization of the Mhb1 protein in alfalfa root tip cells showed the same results (data not shown).

A)

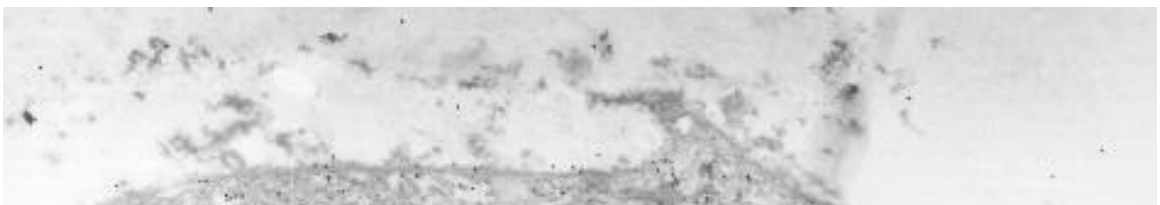


Figure 9 (see next page for legend)

Figure 9 (see previous page)

Immunogold localization of Mhb1 using anti-Mhb1 antibodies in the alfalfa suspension cells

(A) Cross-section of an alfalfa cell from a suspension culture showing mainly nuclear localization of the Mhb1 protein. Magnification: 18000x. Abbreviations for (A) and (B): Nu = nucleus, Cytopl. = cytoplasm, No = nucleolus, Pl = plasmalemma

(B) Immunolabelling carried out with pre- immune serum of the same mouse. Magnification: 18000x.

4.8. Production of transgenic tobacco plants with elevated level of alfalfa non-symbiotic hemoglobin

Using T- DNA- based binary vector constructs, we have produced stable transgenic plants generated by *Agrobacterium*- mediated transformation accumulating the alfalfa non-symbiotic hemoglobin Mhb1. The presence of the Mhb1 protein was confirmed by Western blot analysis based on the polyclonal antibody raised against a recombinant Mhb1 protein (see Section 4.4.). Figure 10 shows that seedlings of the third transgenic generation from selected lines synthesized high amounts of the alfalfa hemoglobin. These transformant plants did not exhibit any obvious phenotypic differences in comparison to the control under standard greenhouse conditions.

4.9. Reduced sensitivity of hemoglobin-overproducing tobacco seedlings towards sodium nitroprusside (SNP) as NO-generating chemical

Similarly to adult plants, no phenotypic or growth rate difference could be seen between 20 μM SNP- treated (data not shown) and control transformant (Figure 11A) and non- transformed (Figure 11B) 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 (Fig. 11C and 11D, respectively) as compared to the untreated ones (Fig. 11A and 11B).

A)

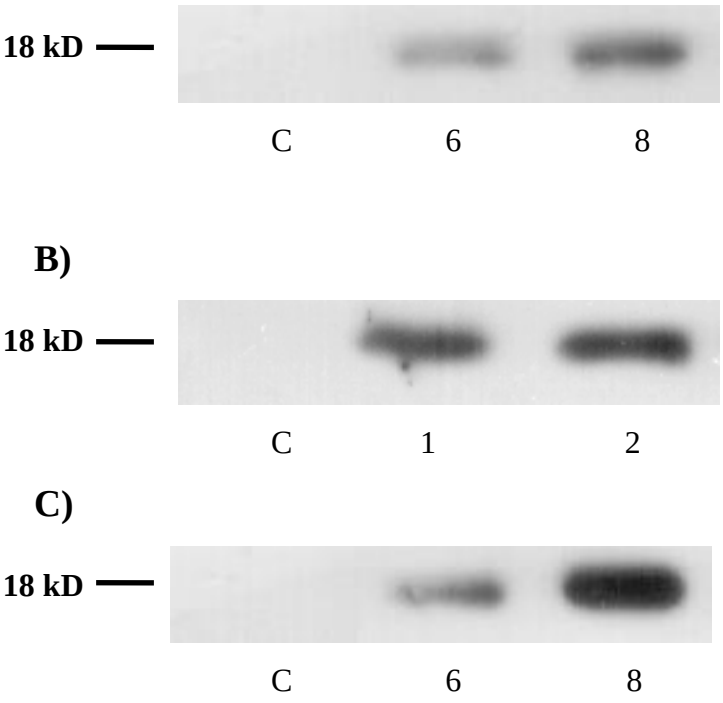


Figure 10

Immunoblot analysis of Mhb1-overproducing tobacco plants

The immunoblots show accumulation of the Mhb1 protein in individual plants from three independent transformant tobacco lines. The polyclonal antibody raised against recombinant Mhb1 protein specifically recognized it at the expected size of 18 kD. C stands for nontransformant SR1 control.
(A) Line 1, plants 6 and 8
(B) Line 11, plants 1 and 2
(C) Line 13, plants 6 and 8

The inhibitory effects were recognizable in delayed organ formation and reduced growth as reflected by fresh weight of seedlings. (Table 1). As demonstrared by photos in Figure 11, 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 (Figures 11C and 11D). We should remark that the seedlings treated with 300 μM SNP did not die, and continued germination at a slower speed.

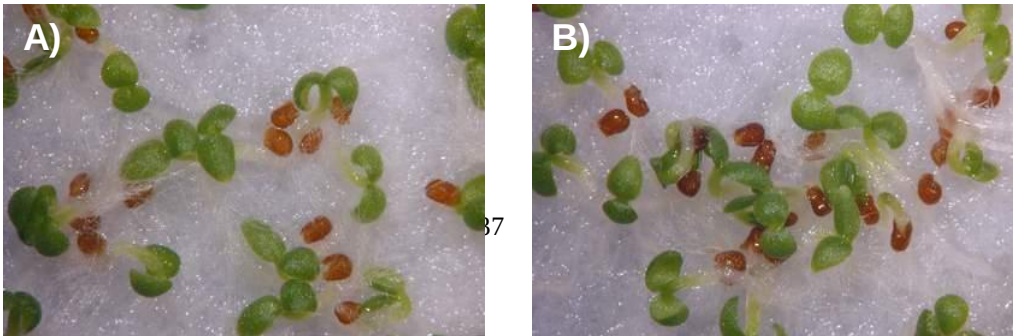


Figure 11

Growth differences during the germination of SR1 and transformed seedlings following treatment with SNP

Photographs of eight day old *Mhb1*-transformant (A, C, E) and non- transformed (B, D, F) tobacco seedlings. Bars represent 5mm.
Untreated seedlings: (A, B)
Seedlings grown on 300 μM of active SNP: (C, D)
Seedlings grown on 300 μM of light-inactivated SNP: (E, F)

Since SNP is a light-sensitive chemical compound, continuous illumination causes the decomposition of SNP while releasing NO. The byproducts of SNP-decomposition could also be responsible for some of the observed growth effects. Therefore we also germinated seeds at

the same concentrations of SNP previously exposed to light to test the role of the unspecific components. When tobacco seedlings were grown with 300 μM of inactivated SNP,

Tobacco lines	Fresh weight of 50 seedlings (mg) upon treatment with active SNP (300 μM)	Fresh weight of 50 seedlings (mg) upon treatment with inactive SNP (300 μM)	Ratio (%)
SR1	11.33±2.29	17,77±3.38	63.75
1	18.33±2.31	22±1	83.33
11	13.33±0.57	13.33±0.57	100.00
13	14±1	18.66±1.15	75.00

Table 1

Growth differences during the germination of SR1 and transformed seedlings following treatment with SNP

significant retardation was observed regardless of the genotype (Figure 11E and 11F). However, the non-transformant seedlings treated with active SNP were significantly smaller than their counterparts treated with the inactivated SNP. The growth differences between the seedlings treated with active and light-inactivated SNP can be attributed to the effect of nitric oxide.

4.10. Mhb1-overexpressing tobacco plants show increased tolerance to sodium nitroprusside (SNP)-induced leaf necrosis

Infiltration of SNP solution into leaf tissues caused extended cell death and systemic spread of necrotic symptoms to non-treated leaf regions (Figure 12). Treatment of adult leaves of control plants with 5 mM SNP solutions (Figure 12B) resulted in severe cell damage. On the basis of comparing responses to inactivated SNP, this damage may be primarily caused by NO. As shown in Figure 12 and Table 2, light-inactivated SNP affected only weakly the treated leaves. Comparing the damages on transformed and control leaves (Figure 12 and Table 2), we observe a significantly smaller necrotic area on leaves of Mhb1-overexpressing

plants after application of active SNP. The differences indicate that accumulation of non-symbiotic hemoglobin may have a protecting effect against NO-related damages.

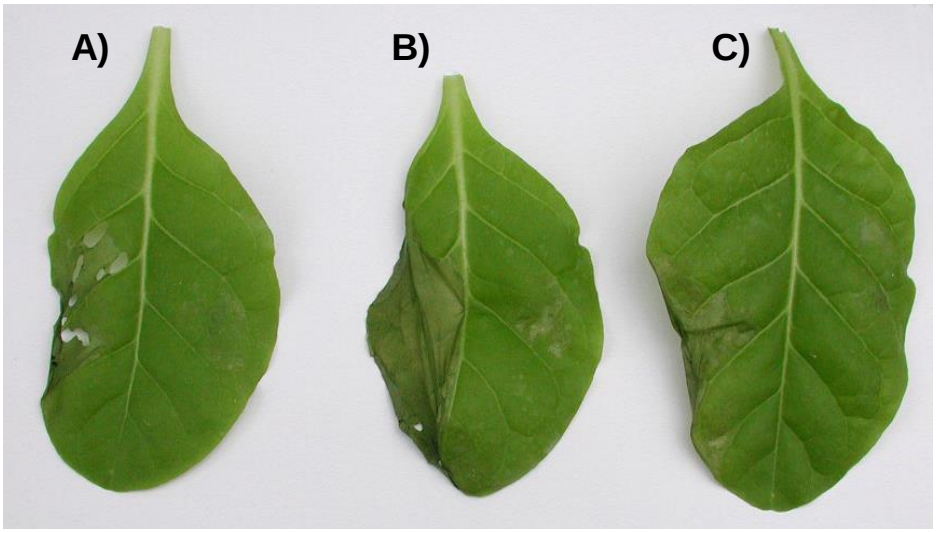


Figure 12

Effect of SNP on mature transgenic and control plant leaves

Damage caused by 5 mM SNP solutions 3 days after injection into the leaves of transgenic (A and C correspond to lines 1 and 11, respectively) and control SR1 (B) tobacco. Half leaves on the left were injected with active SNP solution, half leaves on the right were treated with light-inactivated SNP.

4.11. Tobacco overproducing Mhb1 protein shows reduced symptoms after inoculation with Pseudomonas syringae pv. phaseolicola or TNV

We have injected the bacteria *Pseudomonas syringae* pv. *phaseolicola* in various concentrations into adult transformant and control plant leaves to analyze the extent of damage resulting from hypersensitive necrotisation. Table 3 shows that the hypersensitive response (HR) was suppressed in Mhb1 –overproducing plant leaves as compared to control leaves after inoculation with the bacteria.

Tobacco lines	Inactivated SNP (5mM) Damaged area (mm^2)	Percentage of damage relative to SR1 leaf area damaged by	Active SNP Damaged area (mm^2)	Percentage of damage relative to SR1 leaf area damaged by

		active SNP		active SNP
SR1	53±30	9.6 %	549±232	100 %
1	27±30	4.9 %	199±99	36.2 %
11	23±18	4.2 %	262±144	47.7 %
13	83±67	15.1 %	316±159	57.5 %

Table 2

The reduced necrotization of leaves by active SNP treatment in transformed tobacco plants overexpressing the alfalfa non-symbiotic hemoglobin gene

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 as can be seen in Table 4.

Tobacco lines	$3x10^8$ cm^{-3} bacteria	10^8 cm^{-3} bacteria	$5x10^7$ cm^{-3} bacteria
SR1	4.00±0.00	3.90±0.31	2.00±0.00
11	3.83±0.40	2.60±1.17	1.5±1.91
13	3.66±0.51	2.60±1.34	0.00±0.00

Table 3

Effects of *Pseudomonas syringae* pv. *phaseolicola* infection on SR1 and transformed mature plant leaves

Damage scale:

0 = No symptoms

1 = Just chlorosis

2 = Just a small (2-3 mm in diameter) necrotised area

3 = Diffuse necrotisation of the infected area

4 = The whole infected area is necrotised

Tobacco lines	Number of necrotic lesions/cm^2 of leaf area	Percentage of damage relative to SR1 leaf area damaged by TNV
SR1	3.25±0.40	100%
11	1.32±.09	40.6%
13	1.62±0.55	49.8%

Table 4

Effects of Tobacco Necrosis Virus (TNV) infection on SR1 and Mhb1-overproducing mature plant leaves

Damage scale:

0 = No symptoms

1 = Just chlorosis

2 = Just a small (2-3 mm in diameter) necrotised area

3 = Diffuse necrotisation of the infected area

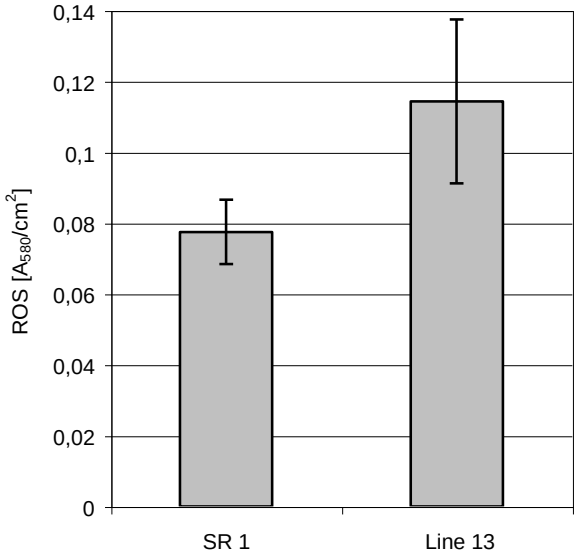
4 = The whole infected area is necrotised

4.12. Mhb1- expressing plants produce more ROS and salicylic acid than control upon infection with Pseudomonas syringae pv. maculicola

To analyze the changes in the ROS and salicylic acid (SA) levels of transgenic and SR1 plants upon pathogen attack, we infected them with 10^8 *Pseudomonas syringae* pv. *maculicola* bacteria. As shown in Figure 13a, the initial ROS levels are higher in the transformed plants. Figure 13b indicates that during infection ROS levels of both lines have increased. However, the similar rate of increase resulted in higher ROS levels in the case of the phytoglobin overproducing line.

Furthermore, free and conjugated salicylic acid levels (SA + SAG) are higher in transgenic tobacco in comparison with SR1 after injection with *Pseudomaonas syringae* pv. *maculicola* (Table 5). Under the same conditions mock infection with 10 mM $MgCl_2$ resulted only in slight differences in the SA-production of transformed and control plants (Table 5).

A)



B)

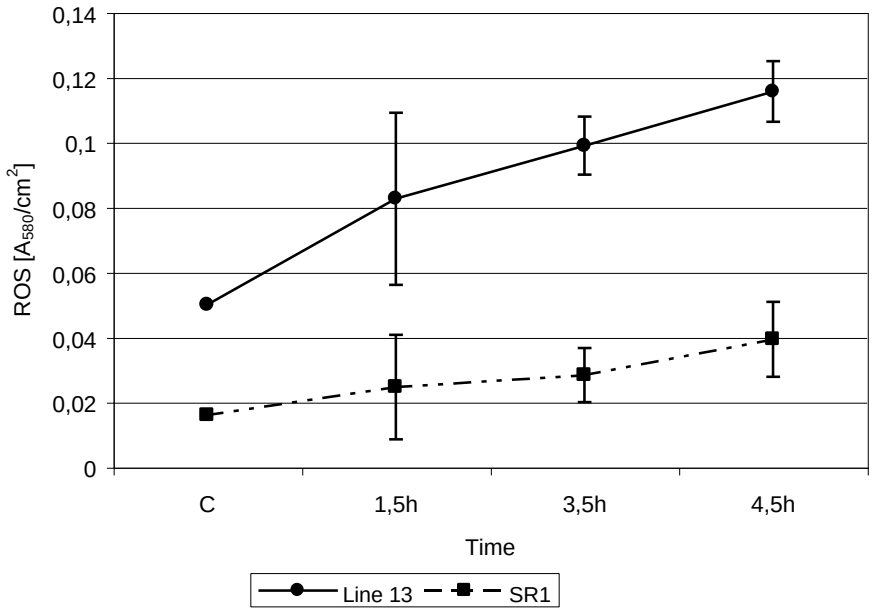


Figure 13

ROS levels in transformed and SR1 plants

(A) ROS levels in untreated transformed (Line 13) and control (SR1) plants (n=3)

(B) Time course analysis of ROS level change in transformant (Line 13) and control (SR1) plants after infection with *Pseudomaonas syringae* pv. maculicola (n=3)

Tobacco lines	SA+SAG concentration in	SA+SAG concentration in

	μg/g fresh leaf weight after mock-treatment (10 mM $MgCl_2$)	μg/g fresh leaf weight after infection with 10^8 cm^{-3} bacteria
SR1	0.96±0.027	1.47±0.078
13_9	1.28±0.14	4.68±0.63

Table 5

Free and conjugated (SA + SAG) SA levels in SR1 and transformed plants before and after infection with *Pseudomonas syringae*, pv. *maculicola*

Chapter 5 – Discussion

The study of non-symbiotic hemoglobins, or phytoglobins, is a relatively novel field of plant biology. This can be seen from how little is really known about phytoglobin function and the many questions based on recently discovered hemoglobin functions in organisms other than plants.

Although phytoglobins have been identified in several plants during the last decade, Mhb1 is the first non-symbiotic hemoglobin that was isolated from alfalfa. Similarly to other phytoglobins, the hypoxia- induced *Mhb1* gene activity was clearly detected on RNA level in cultured alfalfa cells. However, we found that accumulation of the *Mhb1* transcript did not effect Mhb1 protein abundance. This may indicate that the *Mhb1* mRNA is unstable or a higher protein turnover occurs. In the case of higher protein turnover, it is probable that regulatory mechanisms on transcriptional level have important role in keeping Mhb1 protein accumulation on a constant level. The *Mhb1* gene encodes a protein of the class 1 non-symbiotic hemoglobin group according to the classification of phytoglobins of *Arabidopsis thaliana* by Trevaskis *et al.* (1997), in the sense that it can be induced by hypoxia but not by cold stress.

We found that there were changes in the respiration rate during the cell cycle. Out of the two observed respiration rate peaks, the smaller peak at 14 hours coincides with G2/M phases (Figures 7A, and 7C), and the *Mhb1* gene expression peak is just before mitosis (10-12h, Figure 7B), as well. This may imply a link between the increase in metabolic activity required by the mitotic events and higher hemoglobin mRNA levels under non-hypoxic conditions. This may also serve as an indication for the important role of *Mhb1* gene under non-hypoxic conditions of higher metabolic activity as previously suggested by Andersson *et al.* (1996). The correlation of *Mhb1* gene expression with the additional second peak at 20-24 hours, which may correspond to post-mitotic processes such as new

cell wall formation, is also possible, though there is only a slight increase in *Mhb1* mRNA levels during this period.

Since the availability of ATP influences barley phytoglobin expression (Nie and Hill, 1997; Sowa *et al.*, 1998), it is possible that the regulatory mechanism is identical with those mediating hypoxia and the increase in metabolic activity in the sense that there might be a threshold level in the ATP need of the metabolic processes below which non-symbiotic hemoglobin expression is induced in an unknown way.

The observed activation of the *Mhb1* gene at G2/M transition during the cell division cycle in alfalfa suspension cells is also possible during cell divisions taking place in the root tip meristemes and in growing nodules as a result of high metabolic activity. This can be supported by the fact that *Mhb1* mRNA was only found in the roots of alfalfa. In contrast with most phytoglobins, this gene is not detected in other plant tissues. That is why the root induction of *Mhb1* gene can also be explained by the relative hypoxia in the soil.

The result obtained by electronmicroscopic immunolocalization studies can be particularly important if we consider that in maize roots a nitric oxide synthase was localized in the nuclei of elongation zone cells (Ribeiro *et al.*, 1999), though no functional consequnce of this localization has been discovered yet.. Furthermore, the interaction between nitric oxide and hemoglobins in animal cells can contribute to oxygen delivery (Gow and Stamler, 1998). This and the the presence of both of the above mentioned proteins in the nucleus may have a presently unknown functional significance in the nitric oxide signalling pathway.

To analyze the presumable functional role of Mhb1 protein in NO-related signalling with regard to the inhibition of germination, seedling growth and necrotic cell death induced by chemical (SNP) or pathogen treatment, we used Mhb1- expressing transgenic tobacco. The examined transgenic lines were stable enough to express and inherit the transgene properly through at least four generations. The presented data support a significant role for phytoglobins

in reduction of damages caused by the NO-generating compound SNP or infection with viral or bacterial pathogens. The transgenic tobacco plants constitutively synthesizing significant amounts of alfalfa phytoglobin could develop normally both in *in vitro* cultures and in soil under greenhouse conditions. Because many hemoglobins can bind oxygen as well as nitric oxide (Gow and Stamler, 1998; Hausladen *et al.*, 1998), we focused the present study on possible alterations in the NO-responses in Mhb1– expressing transgenic tobacco tobacco plants. Although sodium nitroprusside (SNP) as NO-releasing compound has already been used in several previous experiments with plants (Beligni and Lamattina, 2000; Pedroso *et al.*, 2000; Clarke *et al.*, 2000; Delledonne *et al.*, 1998), this is the first report showing that NO (SNP) induces extended cell death in plant leaf tissues. Here we remark that the present analysis has also clearly revealed some unspecific, inhibitory effects of SNP decomposition byproducts. However, while light-inactivated SNP significantly reduced seedling growth, this side effect was weak during induction of necrotic lesions in adult leaves. This implies that during germination the dividing plant cells can be particularly sensitive towards SNP-treatment. On the other hand, the considerable difference between seedlings treated with active and inactive SNP (Fig. 11) suggest that NO production can inhibit several physiological events during this early stage of plant development. This may relate to the reduction of cell division or the induction of programmed cell death through the activation of a mitogen-activated protein kinase (MAP), as it was observed in *Arabidopsis* cell suspension culture after SNP-treatment by Clarke *et al.* (2000). The role of NO in cell cycle progression is expected to be dependent of the actual concentration of this compound in the cells. The present experimental system has limitations in the tight control of NO level. Therefore, further experiments are needed to reveal the role of NO in cell division control. In spite of the certain unspecific effects of SNP-treatment, the described experiments clearly demonstrated that the presence of elevated phytoglobin level could reduce the inhibitory effects of this NO-generating compound. After treating the seedlings with active

SNP, the transgenic lines showed an improved potential to continue their growth and development. Based on the presented data we propose that phytoglobins can alter the cellular toxicity of NO *in vivo*. This conclusion is in agreement with the protective role of phytoglobin during hypoxia-generated production of NO (Dordas *et al.*, 2002).

A considerable number of publications support a link between NO-signalling and pathogenesis. Inhibitors of nitric oxide synthesis could reduce the hypersensitive reaction of *Arabidopsis* leaves to *Pseudomonas syringae* pv. *maculicola* (Delledonne *et al.*, 1998). On one hand, NO could act synergistically with H_2O_2 and/or with other ROS (Delledonne *et al.*, 1998; Delledonne *et al.*, 2001) and also with salicylic acid in the case of tobacco (Klessig *et al.*, 2000). On the other hand, NO could also act independently of ROS to induce the expression of defense-related genes in the case of *Arabidopsis* cell suspension culture (Clarke *et al.*, 2000) and tobacco (Durner *et al.*, 1998). Furthermore, similarly to the earlier revealed activation of guanylate cyclase by NO in mammals (Arnold *et al.*, 1977), it was also necessary for the NO-induced cGMP-level increase in tobacco (Klessig *et al.*, 2000). We followed two approaches in the phytoglobin effect of NO-elicited responses. Here we describe that injection of SNP into the leaf tissues could generate necrotic symptoms. It is particularly important that SNP-triggered effects could spread to the nontreated leaf regions. Here we can see similarity to the systemic acquired resistance (SAR). This observation is similar to that of Klessig *et al.* (2000) who demonstrated that the formation of SAR is partly under the control of NO. It seems that the SNP action is a specific event, so the released NO is primarily responsible for the symptom development through the induction of cell death. The novel methodology of SNP–infiltration also enabled us to show that phytoglobin sysnthesis could protect differentiated plant cells from the cellular damage caused by NO release. The significance of this molecular defense mechanism was further strengthened by the detection of similar protective role of phytoglobin against different necrotrophic pathogens such as *Pseudomonas syringae* and

Tobacco Necrosis Virus. In attempts to interpret these data, we postulate that the cellular Mhb1 protein can bind NO, therefore the earlier described effect of this gas as a necrotic agent (Delledonne *et al.*, 1998) is reduced.

It has been known that the production of salicylic acid (SA), a key signalling molecule in plant defence against pathogens, can both be NO-dependent (Klessig *et al.*, 2000) and independent. In the latter case SA formation is induced by ROS. (Chamnongpol *et al*., 1998). Furthermore, the interplay between NO- and ROS- triggered defence mechanisms was found necessary for a high efficiency in the protection against pathogen infection (Delledonne *et al.*, 1998). These data strengthened the earlier findings, according to which if we inhibit or eliminate NO, the hypersensitive response (HR) caused by pathogens is suppressed (Delledonne *et al.* 1998, Durner *et al.* 1998). The cited results and our ones suggest that the elevated SA levels observed in the infected transgenic plants can be originated from a compensation event driven by the lack of NO-related defence responses. At the same time, our results also indicate that the interplay between ROS and NO-dependent pathways during plant defence could not be fully substituted by an increase in the SA level of transformant plants.

Delledonne *et al.* (2001) earlier described that one possible reason for the conversion of superoxide to H_2O_2 in soybean was that NO could form peroxynitrite with superoxide very easily. According to their findings, peroxynitrite was much less effective molecule in plant defence than H_2O_2, so with this step the plant chose the more effective defence mechanism. It is known that the nitroblue tetrazolium (NBT) method of ROS determination measures mainly the superoxide level. This may mean that a possible explanation for the higher ROS levels of transgenic plants can be the lack of NO-driven H_2O_2 production as NO is scavenged by the overexpressed Mhb1 phytoglobin.

The present transgenic studies provide a basis to propose that the native phytoglobins synthesized *in planta* either in leaves (Trevaskis *et al.*, 1994) or in vascular tissues (Andersson *et al.*, 1996) can exhibit a protective function after pathogen attack.

The experimental data and their interpretation suggest a common mechanism by which phytoglobins alternate NO- effect both in the cases of SNP-treatment and pathogen infection. It may also be hypothesized that the strict control of intracellular NO level by phytoglobins is involved in the regulation of a variety of NO-related cellular processes.

Chapter 6 – References

Andersson, C.R., Jensen, E.O., Llewellyn, D.J., Dennis, E.S. and Peacock, W.J. (1996) A new hemoglobin gene from soybean: a role for hemoglobin in all plants. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 5682-5687

Appleby, C.A. (1984) Leghemoglobin and rhizobium respiration. *Ann. Rev. Plant Physiol.* **35**, 443-478

Appleby, C.A. (1992) The origin and functions of haemoglobin in plants. *Sci Progress* **76**, 365-398

Appleby, C.A., Tjepkema, J.D. and Trinick, M.J. (1983) Hemoglobin in a nonleguminous plant, *Parasponia*: possible genetic origin and function of nitrogen fixation. *Science* **220**, 951-953

Arredondo-Peter, R., Hargrove, M.S., Sarath, G., Moran, J.F., Lohrman, J., Olson, J.S. and Klucas, R.V. (1997) Rice hemoglobins: gene cloning, analysis and oxygen - binding kinetics of a recombinant protein synthesized in Escherichia coli. *Plant Pysiol.* **115**, 1259-1266

Arredondo-Peter, R., Hargrove, M.S., Moran, J.F., Sarath, G. and Klucas, R.V. (1998) Plant hemoglobins. *Plant Physiol.* **118**, 1121-1125

Beckman, J.S., Chen, J., Ischiropoulos, H. and Crow, J.P. (1994) Oxydative chemistry of peroxynitrite. *Methods Enzymol.* **233**, 229-240

Beligni, M.V. and Lamattina, L. (2000) Nitric oxide evokes light responses in plants. *Planta* **210**, 215-221

Benfey, P.N., Ren, L.and Chua, N-H. (1989) The CaMV 35S enhancer contains at least two domains which can confer different developmental and tissue specific expression patterns. *EMBO J.* **8**, 2195-2202

Bergersen, F.J., Turner, G.L., Appleby, C.A. (1973) Studies of the physiological role of leghaemoglobin in soybean root nodules. *Biochim. Biophys. Acta* **292**, 271-282

Bogusz, D., Appleby, C.A., Landsmann, J., Dennis, E.S., Trinick, M.J. and Peacock, W.J. (1988) Functioning hemoglobin genes in non-nodulating plants. *Nature* **331**, 178-180

Bögre, L., Oláh, Z. and Dudits, D. (1988) Ca^{2+}-dependent protein kinase from alfalfa (Medicago varia): Partial purification and autophosphorilation. *Plant Sci.* **58**, 135-144.

Bradley, D.J., Kjellbom, P. and Lamb, C.J. (1992) Elicitor- and wound-induced oxidative cross-linking of a proline-rich plant cell wall protein: A novel, rapid defense response. *Cell* **70**, 21-30

Bülow, L., Holmberg, N., Lilius, G. and Bailey, J.E. (1999) The metabolic effects of native and transgenic hemoglobins on plants. *Trends Biotech.* **17**, 21-24

Chamnongpol, S., Willekens, H., Moeder, W., Langebartels, C., Sandermann, H. Jr., Van Montagu, M., Inze, D. and Van Camp, W. (1998) Defense activation and enhanced pathogen tolerance induced by H_2O_2 in transgenic tobacco *Proc. Natl. Acad. Sci. U.S.A.* **95**, 5818-5823

Christensen, T., Dennis, E.S., Peacock, W.J., Landsmann, J. and Marcker, K.A. (1991) Hemoglobin genes in non-legumes: cloning and characterisation of a Casuarina glauca hemoglobin gene. *Plant Mol. Biol.* **16**, 339-344

Clarke, A., Desikan, R., Hurst, R.D., Hancock, J.T. and Neill, S.J. (2000) NO way back: nitric oxide and programmed cell death in *Arabidopsis thaliana* suspension cultures. *Plant J.* **24**, 667-677

Delledonne, M., Xia, Y., Dixon, R.A. and Lamb, C. (1998) Nitric oxide functions as a signal in plant disease resistance. *Nature* **394**, 585-588

Delledonne, M., Zeier, J., Marocco, A. and Lamb, C. (2001) Signal interactions between nitric oxide and reactive oxygen intermediates in the plant hypersensitive disease resistance response. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 13454-13459

Doke, N. (1983) Involvement of superoxide anion generation in the hypersensitive response of potato tuber tissues to infection with an incompatible race of Phytophtora infestans and to the hyphal wall components. *Physiol. Plant Pathol.* **23**, 345-357

Dordas, C., Rivoal, J. and Hill, R.D. (2002) Phytoglobins (Nonsymbiotic hemoglobins) and hypoxia. *Annals of Botany* In press.

Duff, S.M.G., Wittenberg, J.B. and Hill, R.D. (1997) Expression, purification, and properties of recombinant barley (*Hordeum sp.*) hemoglobin: Optical spectra and reactions with gaseous ligands. *J. Biol. Chem.* **272**, 16746-16752

Durner, J., Wendehenne, D. and Klessig, D.F. (1998) Defense gene induction in tobacco by nitric oxide, cyclic GMP and Cyclic ADP-ribose. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 10328-33

Durner, J., Gow, A.J., Stamler, J.S. and Glazebrook, J. (1999) Ancient origins of nitric oxide signaling in biological systems. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 14206-14207

Gilles-Gonzalez, M.A., Gonzalez, G., Perutz, M.F., Kiger, L., Marden, M.C. and Poyart, C. (1994) Heme-based sensors, exemplified by the kinase FixL, are a new class of heme protein with distinctive ligand finding and autoxidation. *Biochem.* **33**, 8067-8073

Gow, A.J. and Stamler, J.S. (1998) Reactions between nitric oxide and haemoglobin under physiological conditions. *Nature* **391**, 169-173

Gow, A.J., Luchsinger, B.P., Pawloski, J.R., Singel, D.J. and Stamler, J.S. (1999) The oxyhemoglobin reaction of nitric oxide. *Proc. Natl. Acad. Sci. U. S. A.* **96**, 9027-9032

Hausladen, A., Gow, A.J. and Stamler, J.S. (1998) Nitrosative stress: Metabolic pathway involving the flavohemoglobin. *Proc. Natl. Acad. Sci. U. S. A.* **95**, 14100-14105

Hausladen, A. and Stamler, J.S. (1998) Nitric oxide in plant immunity. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 10345-10347

Hennig, J., Malamy, J., Grynkiewicz, G., Indulski, J., Klessig, D.F. (1993) Interconversion of the salicylic acid signal and its glucoside in tobacco. *Plant J.* **4**, 593-600

Hentze, M.V. and Kühn, L.C. (1996) Molecular control of vertebrate iron metabolism: mRNA-based regulatory circuits operated by iron, nitric oxide and oxidative stress. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 8175-8182

Hill, R.D. (1998) What are hemoglobins doing in plants? *Can. J. Bot.* **76**, 707-712

Holmberg, N., Lilius, G., Bailey, J.E. and Bülow, L. (1997) Transgenic tobacco expressing *Vitreoscilla* hemoglobin exhibits enhanced growth and altered metabolite production. *Nature Biotech.* **15**, 244-247

Horsch, R.B., Fry, J.E., Hoffmann, N.L., Eichholtz, D., Rogers, S.G. and Fraley, R.T. (1985) A simple and general method for transferring genes into plants. *Science* **227**, 1229-1231

Hunt P.W., Watts R.A., Trevaskis B., Llewelyn D.J., Burnell J., Dennis E.S. and Peacock W.J. (2001) Expression and evolution of functionally distinct haemoglobin genes in plants. *Plant Mol. Biol.* **47**, 677-92

Iwaasa H., Takagi T. and Shikama K. (1992) Amino acid sequence of yeast hemoglobin. A two-domain structure. *J Mol Biol*. **227**, 948-954

Jabs, T., Tschöpe, M., Colling, C., Hahlbrock, K and Scheel, D. (1997) Elicitor-stimulated ion fluxes and O2- from the oxidative burst are essential components in triggering defense gene activation and phytoalexin synthesis in parsley. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 4800-4805

Kaló, P., Endre, G., Zimányi, L., Csanádi, G., Kiss, G.B. (2000) *Theor. Appl. Genet.* Construction of an improved linkage map of diploid alfalfa (*Medicago sativa*). **100**, 641-657

Kanayama, Y. and Yamamoto, Y. (1991) Formation of nitrosylleghemoglobin in nodules of nitrate-treated cowpea and pea plants. *Plant Cell Physiol.* **32**, 19-24

Khosla, C. and Bailey, J.E. (1988) Heterologous expression of a bacterial hemoglobin improves the growth properties of recombinant *Escherichia coli*. *Nature* **331**, 633-635

Kim, P.K., Zamora, R., Petrosko, P. and Billiar, T.R. (2001) The regulatory role of nitric oxide in apoptosis. *Int. Immunopharmacol.* **1**, 1421-1441

King, E.O., Ward, M.K. and Raney, D.E. (1954) Two simple media for the demonstration of phycocyanin and fluorescein. *J. Lab. Clin. Med.* **44**, 301-307

Klessig, D.F., Durner, J., Noad, R., Navarre, D., Wendehenne, D., Kumar, D., Zhou, J.M., Shah, J., Zhang, S., Kachroo, P., Trifa, Y., Pontier, D., Lam, E. and Silva, H. (2000) Nitric oxide and salicylic acid signaling in plant defense. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 8849-8855

Kortt, A.A., Inglis, A.S., Fleming, A.I. and Appleby, C.A. (1988) Amino acid sequence of hemoglobin I from root nodules of the non-leguminous Casuarina glauca-Frankia symbiosis. *FEBS Lett.* **231**, 341-346

Kubo, H. (1939) Über Hämprotein aus der Wurzellknölchen von Leguminosen. *Acta Phytochim.* **11**, 195-200

Lamb, C. and Dixon, R. (1997) The oxidative burst in plant disease resistance. *Annu. Rev. Plant Phys. Plant Mol. Biol.* **48**, 251-275

Lander, H.M., Jacovina, A.T., Davis, R.J. and Tauras, J.M. (1996) Differential activation of mitogen-activated protein kinases by nitric oxide species. *J. Biol. Chem.* **271**, 19705-19709

Landsmann, J., Dennis, E.S., Higgins, T.J.V., Appleby, C.A., Kortt, A.A. and Peacock, W.J. (1986) Common evolutionary origin of legume and non-legume plant hemoglobins. *Nature* **324**, 166-168

Leshem, Y.Y., Wills, R.B.H. and Ku, V.V-V. (1998) Evidence for the function of the free radical gas -nitric oxide (NO˙)- as an endogenous maturation and senescence regulating factor in higher plants. *Plant Physiol. Biochem.* **36**, 825-833

Levine, A., Tenhaken, R., Dixon, R.A. and Lamb, C. (1994) H_2O_2 from the oxidative burst orchestrates the plant hypersensitive response. *Cell* **79**, 583-593

Magyar, Z., Bakó, L., Bögre, L., Dedeoglu, D., Kapros, T. and Dudits, D. (1993) Active cdc2 genes and cell cycle phase- specific cdc2- related kinase complexes in hormone-stimulated alfalfa cells. *Plant J.* **4**, 151-161

Malamy, J., Carr, J..P., Klessig, D.F. and Raskin I. (1990) Salicylic acid: a likely endogenous signal in the resistance response of tobacco to viral infection. *Science* **250**, 1002-1004

Malamy, J., Hennig, J. and Klessig, D.F. (1992) Temperature-dependent induction of salicylic acid and its conjugates during the resistance response to tobacco mosiac virus infection. *Plant Cell* **4**, 359-366

McDonald, L. J. and Murad, F. (1995) Nitric oxide and cGMP signaling. *Adv. Pharmacol.* **34**, 263-75

Messmer, U.K., Ankarcrona, M., Nicotera, P. and Brune, B. (1994) p53 expression in nitric oxide-induced apoptosis. *FEBS Lett.* **355**, 23-6

Minning, D.M., Gow, A.J., Bonaventura, J., Braun, R., Dewhirst, M., Goldberg, D.E. and Stamler, J.S. (1999) Ascaris haemoglobin is a nitric oxide-activated 'deoxygenase'. *Nature* **401**, 497-502

Mott, H.R., Carpenter, J.W. and Campbell, S.L. (1997) Structural and functional analysis of a mutant Ras protein that is insensitive to nitric oxide activation. *Biochemistry* **36**, 3640-3644

Murashige, T. and Skoog, F. (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol. Plant.* **15**, 473-497

Mustárdy, L., Los, D.A., Gombos, Z., Murata N. (1996) Immunocytochemical localization of acyl-lipid desaturases in cyanobacterial cells: Evidence that both thylakoid membranes and cytoplasmic membranes are sites of lipid desaturation. *Proc. Natl. Acad. Sci. U.S.A.* **93**. 10524-10527

Nathan, C. and Xie, Q.W. (1994) Nitric oxide synthases: roles, tolls and controls. *Cell*, **78**, 915-918

Navarre, D.A., Wendehenne, D., Durner, J., Noad, R., Klessig, D.F. (2000) Nitric oxide modulates the activity of tobacco aconitase. *Plant Physiol.* **122**, 573-82

Nie, X.Z. and Hill, R.D. (1997) Mitochondrial respiration and hemoglobin gene expression in barley aleurone tissue. *Plant Physiol.* **114**, 835-840

Ota, M., Isogay, Y. and Nishikawa, K. (1997) Structural requirement of highly- conserved residues in globins. *FEBS Lett.* **415**, 129-133

Pedroso, M.C., Magalhaes, J.R. and Durzan, D. (2000) Nitric oxide induces cell death in *Taxus* cells. *Plant Sci.* **157**, 173-180

Poole, R.K. (1994) Oxygen reactions with bacterial oxidases and globins: binding, reduction and regulation. *Antonie van Leeuwenhoek* **65**, 289-310

Poole, R.K., Ioannidis, N., and Orii, Y. (1996) Reactions of the Escherichia coli flavohaemoglobin (Hmp) with NADH and near-micromolar oxygen: oxygen affinity of NADH oxidase. *Microbiology (Reading)* **142**, 1141-1148

Ribeiro, E.A., Cunha, F.Q., Tamashiro, W.M. and Martins, I. S. (1999) Growth phase-dependent subcellular localization of nitric oxide synthase in maize cells. *FEBS Lett.* **445**, 283-286

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989*) Molecular cloning: A laboratory manual*, 2nd ed., Cold Spring Harbor, NY: Cold Spring Harbor Laboratory

Savouré, A., Fehér, A., Kaló, P., Petrovics, G., Csanádi, G., Szécsi, J., Kiss, G., Brown, S., Kondorosi, Á. and Kondorosi, É. (1995) Isolation of a full- length mitotic cyclin cDNA clone *CycIIIMs* from Medicago sativa: chromosomal mapping and expression. *Plant Mol. Biol.* **27**, 1059-1070

Schmidt, H.H.H. W. and Walter, U. (1994) NO at work. *Cell* **78**, 919-925

Soni, R. and Murray, J.A. (1994) Isolation of intact DNA and RNA from plant tissues. *Anal. Biochem.* **218**, 474-476

Sowa, A.W., Duff, S.M.G., Guy, P.A. and Hill, R.D. (1998) Altering hemoglobin levels changes energy status in maize cells under hypoxia. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 10317-10321

Stamler, J. S. (1994) Redox signaling: nitrosylation and related target interactions of nitric oxide. *Cell* **78**, 931-936

Stamler, J.S., Singel, D.J. and Loscalzo, J. (1992) Biochemistry of nitric oxide and its redox-activated forms. *Science* **258**, 1898-902

Stamler, J.S., Jia, L., Eu, J.P., McMahon ,T.J., Demchenko, I.T., Bonaventura, J., Gernert, K. and Piantadosi, C.A. (1997) Blood flow regulation by S-nitrosohemoglobin in the physiological oxygen gradient. *Science* **276**, 2034-2037

Trevaskis, B., Watts, R.A., Andersson, S.R., Llewellyn, D.J., Hargrove, M.S., Olson, J.S., Taylor, E.R., Nie, X.Z., MacGregor, A.W. and Hill, R.D. (1994) A cereal hemoglobin gene is expressed in seed and root tissues under anaerobic conditions. *Plant Mol. Biol.* **24**, 853-862

Trevaskis, B., Watts, R.A., Andersson, C.R., Llewellyn, D.J., Hargrove, M.S., Olson, J.S., Dennis, E.S. and Peacock, W.J. (1997) Two hemoglobin genes in Arabidopsis

thaliana: The evolutionary origins of leghemoglobins. *Proc.Natl. Acad. Sci. U.S.A.* **94**, 12230-12234

Van Camp, W., Van Montagu, M. and Inzé, D. (1998) H_2O_2 and NO: redox signals in disease resistance. *Trends Plant Sci*. **3**, 330-334

Wang, R., Gruegler, K., LaBrie, S.T. and Crawford, N.M. (2000) Genomic analysis of a nutrient response in *Arabidopsis* reveals diverse expression patterns and novel metabolic and potential regulatory genes induced by nitrate. *Plant Cell* **12**, 1491-1510

Watts, R.A., Hunt, P.W., Hvitved, A.N., Hargrove, M.S., Peacock, W.J. and Dennis, E.S. (2001) A hemoglobin from plants homologous to truncated hemoglobins of microorganisms. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 10119-10124

Wendehenne, D., Pugin, A., Klessig, D.F. and Durner, J. (2001) Nitric oxide: comparative synthesis and signaling in animal and plant cells, *Trends Plant Sci*. **6**, 177-183

Wittenberg, J.B. and Wittenberg, B.A. (1990) Mechanisms of cytoplasmic hemoglobin and myoglobin function. *Annu. Rev. Biophys. Chem.* **19**, 217-241

Yun, H.Y., Gonzalez-Zulueta, M., Dawson, V.L., Dawson, T.M. (1998) Nitric oxide mediates N-methyl-D-aspartate receptor-induced activation of p21ras. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 5773-5778

A tézisek összefoglalása magyarul

Bevezetés

A növényi hemoglobinokat tulajdonságaik alapján 3 fő csoportba sorolhatjuk. A legrégebben felfedezett csoportba (1939) a szimbiotikus hemoglobinok tartoznak. Ezek a fehérjék a nitrogénkötő mikroorganizmusokkal szimbiózisban élő növényekben a szimbionta partnert látják el oxigénnel.

A legutóbb (2001-ben) felfedezett növényi hemoglobin-csoportot strukturális sajátságából adódóan „2-on-2 hemoglobins”-nak nevezték el. A csoport idáig egyetlen tagja (*AHB3*) aminosav homológia alapján a mikroorganizmusok hemoglobinjai csonka változatának tekinthető, és hipoxia hatására leszabályozódik.

A harmadik fő csoportot alkotó nemszimbiotikus hemoglobinokat, más néven phytoglobinokat, is sokkal később (a ’80-as években) fedezték fel, mint a szimbiotikusakat. Közös jellemzőjük a szimbiotikus hemoglobinokénál nem ritkán egy nagyságrenddel erősebb O_2-kötő képesség és az, hogy feltehetően minden növényben jelen vannak, nemcsak a szimbiózisban élőkben. Ez utóbbi miatt a nemszimbiotikus hemoglobinokat tekintik ősibbnek és úgy vélik, hogy a szimbiotikus hemoglobinok génduplikáció révén belőlük alakultak ki. Ezt a csoportot két alcsoportra tagolták tovább. Az első alcsoportba a hipoxiára indukálódó gének, a másodikba az előzővel közeli homológiát mutató, de hidegstresszre indukálódó, mostanáig egyetlen ilyen gén tartozik. A közelmúltban publikált adatok alapján a phytoglobinok funkcióját illetően két fő irány határozható meg.

Az egyik szerint a nemszimbiotikus hemoglobin gének akkor indukálódnak, amikor a sejtben az ATP szintje lecsökken. Ennek oka lehet hipoxia, ill. megnövekedett metabolikus aktivitás (pl. sejtosztódás a merisztémában) is. Pl. egy kísérlet szerint részleges hipoxiás (5%

O_2) kezelést követően az árpa phytoglobint túltermelő kukorica sejtszuszpenzió ATP szintje a kontrollénál kb. 30%-kal magasabbnak bizonyult.

A másik funkció egyrészt akkor körvonalazódott, amikor tanulmányok jelentek meg a nitrogén-monoxid (NO) különböző szerepeiről a növény életében, különös tekintettel a növényi patogének elleni védekezési reakcióban betöltött funkciójára. Másrészt pedig akkor, amikor világossá vált, hogy az egysejtűektől a magasabbrendű állati szervezetekig mindenütt nagy jelentőséggel bír a növényekben a közelmúltban is csak részben leírt kölcsönhatás az NO és a hemoglobinok között. Az említett reakciót növényekben hipoxiának kitett gyökerekben történő NO-termelődés után figyelték meg először (2002), mivel a nemszimbiotikus hemoglobint túltermelő gyökerekben az NO szintje lecsökkent a kontroll növényekben mért értékekhez képest. Jelen esetben a phytoglobinnak néhány mikroorganizmus hemoglobinjához hasonlatos detoxifikáló funkciót tulajdonítottak, mivel feltételezhető volt, hogy az oxihemoglobin az NO-t NO_3^--á oxidálta tovább, ami növények esetében kevésbé toxikus és jobban felhasználható vegyület. Emellett a nitrozilhemoglobin (NO kötődése a hemhez) képződése is csökkentheti a sejthalált is előidézni képes NO szintjét.

Mivel a nemszimbiotikus hemoglobinok termelődését a gyökéren kívül más növényi szövetben is megfigyelték (pl. szárban, rozettalevélben), ezért feltételezik, hogy nemcsak a gyökérben és nemcsak hipoxia alatt hathat köcsön az NO a phytoglobinokkal, hanem pl. a patogének elleni válaszreakció során e kölcsönhatás képes befolyásolni az NO-függő jelátviteli mechanizmust is, megváltoztatva a növény védekezőképességét. Ezért helyezett jelen tanulmány is nagy hangsúlyt az NO és a phytoglobinok *in vivo* kölcsönhatásának és az ebből adódó következmények vizsgálatára.

Célkitűzések

Célul tűztük ki az általunk izolált *Mhb1* gén molekuláris és funkcionális jellemzését, mivel a phytoglobinok szerepe a növény életében még tisztázásra vár. Ennek érdekében a következő kérdésekre kerestünk választ.

I. Az *Mhb1* gén és termékének molekuláris jellemzése:

1. Mely lucerna szövetekben expresszálódik az *Mhb1* gén?
2. Indukálható-e az *Mhb1* gén transzkripciója a többi nemszimbiotikus hemoglobin esetében korábban már leírt módon (hipoxia, hidegstressz)?
3. Van-e összefüggés a sejtek metabolikus aktivitásának megváltozása és az *Mhb1* gén indukciója között?
4. Milyen egyéb olyan tulajdonsággal rendelkezik az *Mhb1* gén ill. fehérje, amely további bizonyítékul szolgál a funkciójára?

II. Mhb1 fehérjét túltermelő transzformáns dohánynövények létrehozása és vizsgálata

1. Van-e különbség a transzformánsok és a kontroll növények fenotípusa között normál körülmények esetén?
2. Van-e lehetőség egyéb módon különbséget tenni a transzformáns és transzformálatlan növények között az Mhb1 fehérje működése szempontjából, különös tekintettel annak NO-val való kölcsönhatására?

Eredmények

Lucerna sejtszuszpenzióból készített cDNS könyvtárból izoláltunk egy 483 bp-ból álló, teljes hosszúságú cDNS klónt (*Mhb1,* GenBank azonosító: AF172172), ami egy 160 as. hosszúságú nemszimbiotikus hemoglobint kódol, és tartalmaz minden, a növényi hemoglobinokra jellemző aminosav-motívumot. Kimutattuk, hogy az *Mhb1* gén 1 kópiában van jelen a genomban.

Northern analízissel megállapítottuk, hogy az *Mhb1* mRNS-t felnőtt lucernanövény gyökerében van csak jelen. Kis mennyiségben normál körülmények között tartott lucerna sejtszuszpenzióban is kimutattuk a jelenlétét, amely hipoxia hatására jelentősen megemelkedett.

Az *Mhb1* gén indukcióját észleltük közvetlenül a G2/M fázis előtt és részben a G1-ben is, szinkronizált lucerna sejtszuszpenzióból izolált mRNS Northern analízise után. Továbbá megállapítottuk, hogy ugyanennek a szinkronizált sejtszuszpenziónak az O_2-fogyasztása G2/M-ben kb. 20, míg G1-ben kb. 50%-kal magasabb egy szinkronizálaltlan sejtszuszpenzión mért átlagértéknél.

A rekombináns Mhb1 fehérje ellen létrehozott poliklonális ellenanyag segítségével kimutattuk, hogy hipoxia vagy hidegstressz hatására az Mhb1 fehérje szintje lucerna sejtszuszpenzióban nem változott a kezeletlen szuszpenzió Mhb1-szintjéhez képest. Mivel az elleanyag ezt a 18 kDa tömegű fehérjét specifikusan ismerte fel, ezért elektronmikroszkópos immunolokalizációt is végrehajtottunk a szuszpenziósejteken. Ennek eredményeként megtudtuk, hogy az Mhb1 fehérje főként a sejtmagban (de nem a magvacskában), kisebb mértékben pedig a citoplazmában található.

Olyan transzformáns növényeket hoztunk létre, melyek az Mhb1 gént konstitutívan expresszálják a CaMV35S promóter segítségével. A túltermelt Mhb1 fehérje jelenlétét immunoblottal ellerőriztük A transzformánsok fenotípusa nem tért el a kontroll növényekétől. A kísérletekhez harmadik generációs transzformáns növények magjait használtuk.

Harmadik generációs transzformánsokból és kontroll növényekből származó magokat csíráztattunk és 300 μM NO-donor vegyülettel (SNP) kezeltük őket. A transzformánsok a kezelés hatására kevésbé maradtak le a növekedésben, bár a vegyületnek jelentős és kb. azonos aspecifikus hatása is volt mind a transzformánsokra, mind a kontrollra nézve.

Kifejlett transzformáns és kontroll növények levelét 5 mM SNP-vel kezelve a transzformáns levelek jelentősen kisebb mértékben nekrotizálódtak, mint a kontroll. A kifejlett növények levelén a csírákhoz képest jelentősen kisebb aspecifikus SNP-hatást láttunk.

Kifejlett transzformáns és kontroll növények levelét baktériummal (*Pseudomonas syringae* pv. *phaseolicola*) vagy vírussal (TNV) fertőzve a transzormáns növények levelei kisebb mértékben haltak el, mint a kontrollei.

Baktériummal (Pseudomonas syringae) való fertőzést követően az Mhb1-túltermelő növények levelében mért reaktív oxigén (ROS) és szalicilsav (SA)-szint a kontrollénál nagyobb mértékben emelkedett úgy, hogy már a fertőzés előtt mért ROS és SA szintek is meghaladták a kontrollban mértekét.

A fenti eredményekből az alábbi következtetéseket vonhatjuk le:

- Az *Mhb1* gén a kifeljett lucerna növénynek csak a gyökerében expresszálódik, aminek egy lehetséges oka a gyökeret érő relatív hipoxia.
- Az *Mhb1* gén lucerna sejtszuszpenzióban a többi, 1. csoportba tartozó nemszimbiotikus hemoglobinhoz hasonlóan hipoxiára indukálódott, bár az Mhb1 fehérje szintje nem változott meg. Ennek oka lehet az Mhb1 fehérje magasabb turnovere, vagy az instabil *Mhb1* mRNS is.
- Az *Mhb1* gén G2/M előtt és G1-ben tapasztalt indukciója jól korrelál ugyanazon szinkronizált sejtszuszpenzió O_2-fogyasztásának változásával. Ez a sejtosztódás szintjén támasztja alá azt a korábbi feltételezést, hogy a nemszimbiotikus hemoglobinok képesek a metabolikusan aktív szövetekben indukálódni. Továbbá a gyökérben tapasztalt *Mhb1* mRNS-szint is magyarázható ezzel a jelenséggel, mert

a lucerna növény gyökere viszonylag sok osztódó szövetet (gümők, gyökércsúcsok) tartalmaz.

- Az NO sejtosztódást és fejlődést gátló kisebb hatása a transzformáns csírákra az NO-nak a túltermelt Mhb1 fehérjéhez való kötődésével és így az aktív NO koncentrációjának csökkenésével magyarázható.
- Ugyanezt a mechanizmust valószínűsíti a kifejlett transzformáns növények már differenciálódott leveleinek SNP-kezelést követő kisebb mértékű nekrózisa a kontrollhoz képest. Ugyanitt megfigyeltük az elhalás szisztemikus átterjedését a nem kezelt területekre is.
- Szintén az NO-hemoglobin kölcsönhatás mérsékelheti a patogének támadásakor aktiválódó NO-függő jelátviteli mechanizmus hatékonyságát, amely egyébként jelentős alkotóeleme a növényi immunválasznak.
- Ezt támasztja alá a fertőzött transzformáns leveleken mért magasabb ROS és SA-szint is, amely valószínűleg a NO-függő jelátviteli mechanizmus részbeni kiesése miatt van magasabb szinten baktérium-fertőzés előtt és azt követően is. Azonban a sejtek az NO-tól csak részben független védekezési utak megerősítésével vélhetően nem teljesen képesek kompenzálni az NO hiányát.

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