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NO-degradation by alfalfa class 1 hemoglobin (Mhb1): a possible link to *PR-1a* gene expression in Mhb1-overproducing tobacco plants

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Abstract **Tobacco plants overproducing alfalfa class 1 hemoglobin (HOT plants) have been shown to have reduced necrotic symptom development. Here, we show that this altered pathogenic response is linked to a significant increase in the nitric oxide (NO)-affected pathogenesis-related (*PR-1a*) transcript accumulation in the transgenic plants. Homogenates of HOT transgenic seedlings were also found to have higher NO-scavenging activity than non-transformed ones. The NO-scavenging properties of recombinant alfalfa class1 hemoglobin have been examined. Recombinant Mhb1 (rMhb1) was produced in bacteria and purified using polyethylene glycol (10–25%) fractionation, chromatography on DEAE–Sephacel, and Phenyl Superose columns. After the final purification step, the obtained preparations were near homogeneous and had a molecular weight of 44 kDa determined by size-exclusion chromatography and 23 kDa by SDS–PAGE, indicating that rMhb1 is a dimer. The protein participated in NO-degradation activity with NAD(P)H as a cofactor. After ion-exchange columns, addition of FAD was necessary for exhibiting maximal NO-degradation activity. The NAD(P)H-dependent NO-scavenging activity of rMhb1, which is similar to that of barley hemoglobin, supports a conclusion that both monocot and dicot class 1 hemoglobins can affect cellular NO levels by scavenging NO formed during hypoxia, pathogen attack and other stresses.**
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Keywords: Class1 hemoglobin; Nitric oxide; Purification; Hexacoordinate; Pathogen; *PR-1a*

1. Introduction

Nitric oxide (NO) is an important factor in the response to pathogen infection [1,2]. The response is believed to be through a cGMP-mediated induction of early and late defense reactions. During this event, NO induces guanylate cyclase and the cGMP produced can increase salicylic acid (SA) formation via Ca^{2+}-mediated signalling, resulting in the activation of such defense genes as phenylalanine-ammonia lyase or pathogenesis-related 1 (*PR-1a*) [1]. The burst of reactive oxygen species (ROS) that occurs after pathogen attack during the hypersensitive response (HR) [3] is also known to induce SA production [4], suggesting that ROS can trigger *PR-1a* expression via an effect on SA production.

Recent findings support the hypothesis that plant (class 1) hemoglobins can interfere with NO-related processes. In tobacco plants, overproduced alfalfa class 1 hemoglobin (Mhb1) can reduce necrotic symptoms compared to non-transformed control after either treatment with an NO-donor sodium nitroprusside (SNP) or infection with viral and bacterial pathogens [5]. Alfalfa root cultures overexpressing barley hemoglobin accumulate less NO than either control or antisense barley hemoglobin alfalfa lines during hypoxic treatment [6]. Cultured alfalfa roots expressing sense or antisense barley hemoglobin have been shown to scavenge NO by converting it to nitrate [7]. As it has been shown that different hemoglobins can bind and scavenge NO [8,9], the influence of class 1 hemoglobins on NO-metabolism was attributed to an NO-hemoglobin interaction.

Class 1 hemoglobins are known to possess ligand binding characteristics different from their symbiotic counterparts. While symbiotic hemoglobins have lower oxygen affinity and fast oxygen dissociation rates to facilitate O_2 diffusion [10–12], class 1 hemoglobins have high oxygen affinities and low oxygen dissociation rate constants [13–15]. This implies that class 1 hemoglobins may have functions other than transport of O_2. This difference in ligand binding originates from the difference in the heme coordination of these proteins [13,16,17]. Class 1 hemoglobins proved to be hexacoordinate (as opposed to pentacoordinate), referring to the number of bound heme coordination sites in the absence of oxygen. Such hexacoordinate hemoglobins exist throughout the animal and plant kingdoms, in *Synechocystis* [18], *Chlamydomonas* [19], *Drosophila* [20] and in humans (cytoglobin [21] and neuroglobin [22]), but their function is still unclear.

Here, we demonstrate that NO-degradation activity is partially retained during the purification of recombinant alfalfa class 1 hemoglobin [23], and this activity is NAD(P)H- and FAD-dependent. This can correspond to earlier suggestions about plant class 1 hemoglobin-NO interaction [24,25], and may imply a possible role of hemoglobin in NO-involved signalling mechanisms, such as pathogen infection. We also present that NO degradation function was significantly higher in homogenates of transgenic tobacco seedlings previously shown to overexpress Mhb1 compared to non-transformed

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control. We report that the induction of the pathogenesis-related gene *PR-1a* was found to be elevated upon pathogen attack in tobacco plants overproducing Mhb1 protein. A potential link of this response to the earlier shown increase in other NO-related signalling components, such as ROS and SA [5], is also discussed.

2. Materials and methods

2.1. Plant material and homogenization

Tobacco plants (*Nicotiana tabacum* cv. Petit Havanna line SR1) were used to obtain lines overproducing Mhb1 protein (HOT plants) as described in [5]. Two month old SR1 and HOT plants were infected with *Pseudomonas syringae* pv. *maculicola* at a concentration of 10^8 cfu/ml as described in [5]. Homogenates of control SR1 and HOT plants were prepared from 8 day old seedlings by grinding them under liquid N_2 and using 50 mM Tris–HCl, pH 7.5, as extraction buffer.

2.2. Protein production

Mhb1 cDNA was cloned into the *Bam*HI–*Xho*I restriction sites of the pTRCHis B expression vector (Invitrogen, Carlsbad, USA) polylinker region. The resulting polyhistidine-tagged recombinant Mhb1 (rMhb1) protein was 44 amino acids (aa) longer than its native counterpart. This is partly because of the histidine tag (31 aa) and partly because of 13 extra amino acids upstream of the Mhb1 protein original start codon (NH_2-DPFVAVNINTLEN-COOH) because of the cloning procedure. This resulted in the ca. 23 kDa predicted molecular weight of the recombinant monomer as compared to its original weight of 18 kDa based on calculations from the deduced amino acid sequence [23]. Since the additional N-terminal amino acids do not contain any prosthetic groups that could interact with NO, we assume that the observed NO-scavenging originates from the hemoglobin part of the recombinant protein.

2.3. Extraction and purification of recombinant alfalfa hemoglobin

All procedures were performed at 4 °C and all chromatographic separations were done on a Pharmacia FPLC protein purification system. All buffers were degassed at 20 °C. Washed cells were resuspended (5 g/40 ml) in extraction buffer (50 mM Tris–HCl, pH 8, 100 mM NaCl, 10% (w/v) sucrose, 1 mM EDTA and 20% (v/v) glycerol). Cells were disrupted by two passes through a chilled French pressure cell at approximately 20 000 psi. The lysate was clarified by centrifugation at $27\,000 \times g$ for 10 min. The supernatant fluid was then fractionated with 10% and then 25% polyethylene glycol (PEG) 8000.

The red coloured 10–25% PEG pellet was redissolved in 30 ml of buffer A (50 mM Tris–HCl, pH 8.5 and 1 mM EDTA) containing 10% glycerol and the clarified solution was applied at a rate of 1 ml/min to a DEAE–Sephacel column preequilibrated with buffer A. After a thorough washing, the protein was eluted with a 100 ml linear gradient of 0–500 mM NaCl in buffer A.

The fractions containing the most red colour were pooled and made to 30% saturation with $(NH_4)_2SO_4$ and dissolved. The sample was then loaded onto a Phenyl Superose column equilibrated with buffer A containing 30% $(NH_4)_2SO_4$. Mhb1 was then eluted at a flow rate of 1 ml/min with a 50 ml linear gradient of 30–0% $(NH_4)_2SO_4$ in buffer A. Fractions were analyzed by absorbance, and those containing the most Hb relative to total protein were pooled and concentrated to a final volume of approx. 200 μl and buffer exchanged into PBS (40 mM KH_2PO_4/K_2HPO_4, pH 7.0 and 150 mM NaCl) using a Centricon 10 concentrator (Millipore, Bedford, USA). The purified hemoglobin was then either immediately used for analysis or stored at −80 °C until needed.

2.4. Molecular mass determination

The native molecular mass of the recombinant alfalfa hemoglobin was determined by size exclusion chromatography on a Superose 12 column using 50 mM Tris–HCl, pH 8.5, and 100 mM NaCl as column buffer. Fractions (0.5 ml) were collected with a flow rate of 0.5 ml/min and assayed for A_{280} and A_{412}.

2.5. Measurement of NO conversion by hemoglobin

NO conversion was measured amperometrically using an NO electrode (NOMK2, World Precision Instruments, USA) in 2 ml of 50 mM Tris–HCl buffer (pH 7.4) containing 1 mg/ml bovine Cu,Zn-superoxide dismutase (Sigma) to prevent formation of peroxynitrite [26] and, after ion-exchange chromatography, 5 μM FAD. NO was continuously monitored and the NO degradation rate measured from the slope of the NO vs. time curve. For NO production, 1 mM SNP was added with continuous stirring, the vial was illuminated to produce NO at a saturating concentration of 1–1.5 μM. The sample (1–10 μl) was added followed by the addition of NAD(P)H (0.1 mM). There was no NO decrease with NAD(P)H in the absence of the sample. While testing diphenylene iodonium (DPI), the sample was preincubated for 10 min with the inhibitor before adding NADH. Total protein was determined by the method of Bradford [27].

For NO conversion by HOT and control (SR1) seedling homogenates, 5 μl of homogenate was added to the buffer used for measuring NO-conversion.

2.6. Electrophoresis and mass spectrometry

SDS–PAGE electrophoresis has been performed using BioRad Mini-Protean II system with acrylamide concentration of 15%. Proteins were stained with Coomassie brilliant blue R-250 and the band of alfalfa hemoglobin was cut out and used for mass-spectrometry. Coomassie stained band was in situ digested with modified trypsin as described in [28].

MS-MS analysis of the peptide mixture was performed by MALDI Qq-TOF mass spectrometer (Manitoba/Sciex prototype) [29,30] at Manitoba Proteomics Centre (Winnipeg, Canada). Tandem MS spectra were analyzed by using the software package *m/z* (Proteometrics Ltd., New York, USA) and Sonar MS-MS (Proteometrics Canada) search engine.

2.7. Semi-quantitative RT-PCR assay

RNA was isolated with Trizol reagent according to the method of Chomczynski [31] and then treated with DNase according to Promega (Wisconsin, USA) protocol. 1 μg of RNA from each sample was used for the reverse transcription reaction in 20 μl with oligo (dT)18 primer and M-MuLV reverse transcriptase. Reaction was performed according to MBI Fermentas Gmbh (St. Leon-Rot, Germany) protocol for first-strand cDNA synthesis. One and a half microliters of the reverse transcription reaction (0.075 μg RNA of template) was used for PCR analysis of *PR-1a* or one microliter of the reverse transcription reaction (0.05 μg RNA of template) was used for PCR analysis of elongation factor 1 alpha (*EF*1α). PCR amplifications were done in a 25 μl volume containing 1× PCR buffer with cDNA, 0.4 μM specific primers (5′-CACAATTGCCTTCATTTCTTC-3′ 5′-CTAGCACATCCAACACGAAC-3′ /*PR-1a*/) or (5′-TCACATCAACATTGTGGTCATTGGC-3′ 5′-TTGATCTGGTCAAGAGCCTCAAG-3′/*EF*1α/), 0.25 mM dNTPs, 2.5 mM $MgCl_2$, and 1.0 unit of Taq DNA polymerase (MBI Fermentas, St. Leon-Rot, Germany). The PCRs were run in a GeneAmp 2400 PCR System (Perkin–Elmer, California, USA) and the products were analyzed by 0.8% agarose gel elecrophoresis with ethidium bromide staining.

2.8. Quantitative real-time PCR assay

Amplification, data acquisition, and data analysis were carried out using GeneAmp 5700 Sequence Detection System (Applied Biosystem, California, USA). To amplify plant DNA, the real-time PCR used double-stranded DNA-specific dye SYBER Green I. The reaction mixture contained template DNA, 12.5 μl 2× QuantiTect SYBER Green PCR Master Mix (QIAGEN Gmbh, Hilden, Germany), and 50 nM primers in a final volume of 25 μl. PCRs were cycled using the following parameters: 15 min initial denaturation at 95 °C and 35 cycles of 94 °C for 20 s, 59 °C for 20 s and 72 °C for 20 s. Template negative reactions were also included in all amplification experiments.

3. Results

3.1. Purification of recombinant alfalfa hemoglobin using FPLC

The purification of recombinant alfalfa hemoglobin is shown in Table 1. The protein was purified to a high final purity (after size exclusion chromatography) as assessed by SDS–PAGE (Fig. 1). The protein has a molecular mass of ca. 23 kDa

Table 1
Purification of Mhb1 recombinant protein produced by *E. coli*

Fraction	Volume (ml)	Total protein (mg)	Total rMhb1 (mg)	Degree of rMhb1 purification	Total NO-scavenging activity (nmol NO/min)	Specific NO-scavenging activity (nmol NO/min/mg protein)
Total extract	55	764.5	48.93	1	42 765	55.94
10–25% PEG redissolved pellet	30	285.3	30.53	1.67	6055	21.22
DEAE–Sephacel	16	28	5.6	3.12	2490	88.93
Phenyl Superose	4	1.564	1.030	10.28	330	211
Superose 12	0.1	0.0938	0.0856	14.26	1.74	18.55

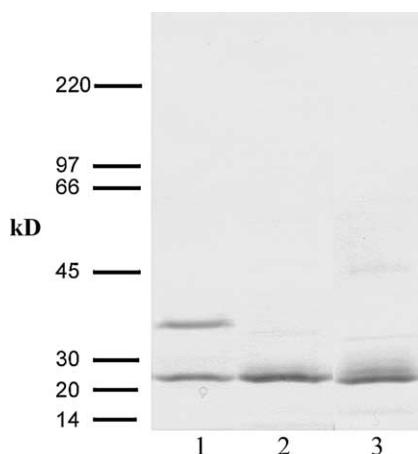

Fig. 1. SDS–PAGE showing three stages of purification (each lane contains 8 μg of protein). Lane 1: sample after DEAE–Sephacel column. Lane 2: sample after Phenyl Superose column. Lane 3: sample after size-exclusion (Superose 12) column.

determined by SDS–PAGE (Fig. 1), which is about 5 kDa higher than the molecular weight of the native Mhb1 protein (based on calculations from the deduced amino acid sequence [23]). This corresponds to the expected amino acid sequence of the recombinant hemoglobin, which is 44 aa longer than its native counterpart.

Molecular mass of the recombinant protein was determined to be 44 ± 4 kDa by size exclusion chromatography on a Superose 12 column. This suggests that the Mhb1 protein is a homodimer, similarly to other plant class 1 hemoglobins.

3.2. NO-degrading activities of alfalfa class 1 hemoglobin

Table 1 shows both total and specific NO-scavenging activities of the recombinant alfalfa hemoglobin along with its degree of purification after each step. A rapid loss of total NO-scavenging activity can be observed throughout the purification, yet there is a 4-fold increase in the specific NO-scavenging activity up to the point of purification on the Superose 12 column. After the Phenyl Superose step, the activity to degrade NO was still high in the fraction following the hemoglobin peak while hemoglobin concentration at that point decreased by about 10-fold (Fig. 2). The specific activity after the Superose 12 column decreased by about 90% and corresponded to 18.5 nmol/min/mg protein (Table 1). To scavenge NO, addition of either NADH or NADPH was required.

Mass spectrometric analysis of the protein band obtained after purification on the Phenyl Superose column showed that it was highly homologous to the native alfalfa class 1 hemoglobin protein sequence (data not shown).

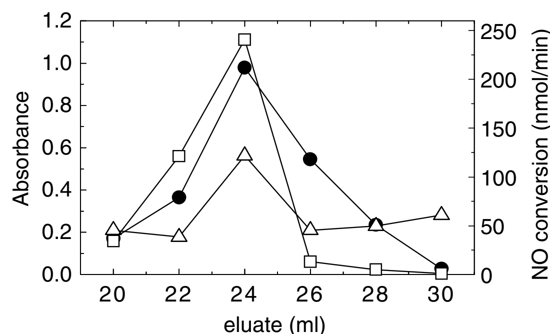

Fig. 2. Elution profiles from Phenyl Superose column of total protein (A_{280}, open triangles), hemoglobin (A_{412}, open squares) and the ability of the fraction to degrade NO, expressed as NO conversion activity (black circles).

Activity data for the untransformed bacterial extract (lacking recombinant Mhb1 protein) exhibit a dramatic loss both in total and specific activities of NO-scavenging by the end of the second purification step (PEG fraction, Table 2), even though NADH was added at a final concentration of 0.1 mM.

To regenerate oxyhemoglobin from the methemoglobin form, FAD (at a final concentration of 5 μM) was also added to the NO-scavenging (Fig. 2) and regeneration (Fig. 3) experiments following purification on DEAE–Sephacel and Phenyl Superose columns. No significant rate of oxyhemoglobin regeneration from methemoglobin was observed in the presence of NADH without added FAD. Activity in the presence of NADPH instead of NADH was about $50 \pm 10\%$ of the original rate. The NO-degradation by Mhb1-containing fractions from the Phenyl Superose column could be completely inhibited by 10 μM diphenylene-iodonium (DPI, data not shown).

Homogenates from Mhb1-transgenic tobacco seedlings (HOT 11 and HOT 13 lines) showed increased rates of NADH-dependent NO-scavenging compared to non-transformed SR1 seedlings (Table 3).

3.3. RT-PCR and real-time PCR of *PR-1a* gene expression in HOT plants upon bacterial infection

Tobacco lines overexpressing alfalfa class 1 hemoglobin (HOT 1, HOT 11, and HOT 13 [5]) were infected with *P. syringae* pv. *maculicola* (Psm) suspension at a concentration of 10^8/ml. Fig. 4a presents pathogenesis-related (*PR-1a*) gene expression levels quantitated by real time PCR assay before and after inoculation of the bacteria or mock inoculation. The *PR-1a* gene expression level in Mhb-transgenic and control leaves was very low and only little change was observed before 12 hpi. In contrast, at 18 hpi, *PR-1a* level in *Mhb1*-transgenic increased

Table 2
NO-scavenging activity in fractions from non-transformed *E. coli*

Fraction	Volume (ml)	Total protein (mg)	Total activity (nmol NO/min)	Specific activity (nmol NO/min/mg protein)
Total extract	22.88	255.3	9848	38.57
10–25% PEG pellet	12	75.48	144	1.90
DEAE–Sephacel	4.5	1.66	0	0

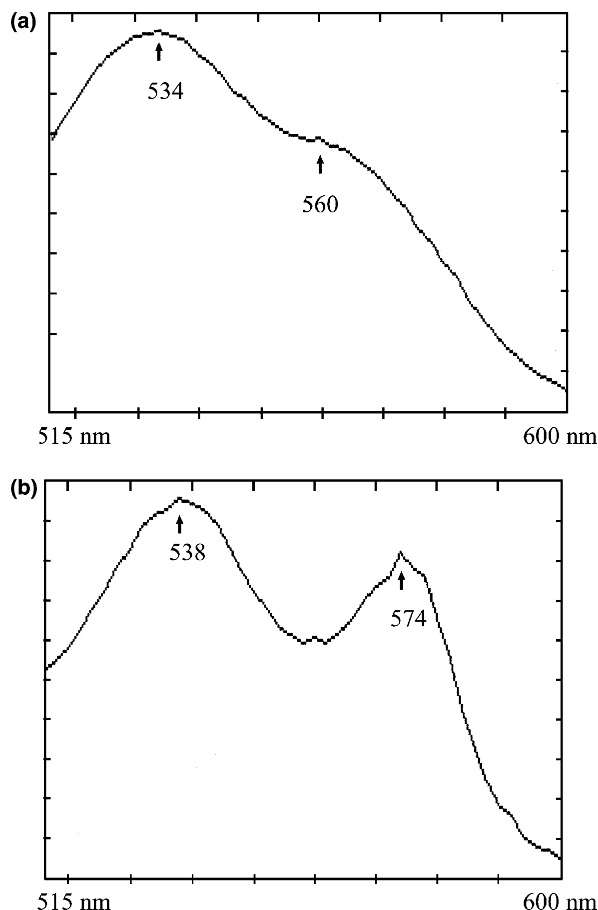

Fig. 3. Spectra of purified recombinant alfalfa hemoglobin. (a) Ferric methemoglobin form; (b) formation of oxyhemoglobin after incubation with 0.1 mM NADH and 5 μM FAD. Absorption maxima of the forms, corresponding to those obtained for barley hemoglobin [15], are indicated by arrows.

Table 3
NO-conversion activities of seedlings

Tobacco lines	Rate of NO-scavenging (μmol NO/min/g FW)	Ratio (%)
SR1	0.173 ± 0.004	100
HOT 11	0.205 ± 0.010	118
HOT 13	0.274 ± 0.030	158

approximately 10-fold (Fig. 4a) and was higher compared to control plants. Finally, at 24 hpi, *PR-1a* concentration in Mhb-transgenic lines was 3-times higher compared to control plants (Fig. 4a). The observed difference in the expression of *PR-1a* gene was repeated in two independent experiments.

Furthermore, semi-quantitative RT-PCR analysis of *PR-1a* gene expression also shows an increase after infection in leaves of Mhb1-transgenic lines compared to non-transformed SR1 plants (Fig. 4b). Differences in transcript expression of the *PR-1a* gene between *Mhb1*-transgenic HOT lines and SR1 lines were first detected at 12 h postinfection (hpi). Differences in *PR-1a* gene expression between HOT and SR1 lines were still detectable up to 24 hpi. A similar time course of *PR-1a* gene expression was observed in another Mhb1-transgenic line (HOT 1 line [5] – data not shown). *PR-1a* gene induction was not observed during mock-infection of transgenic or control plants (Fig. 4b).

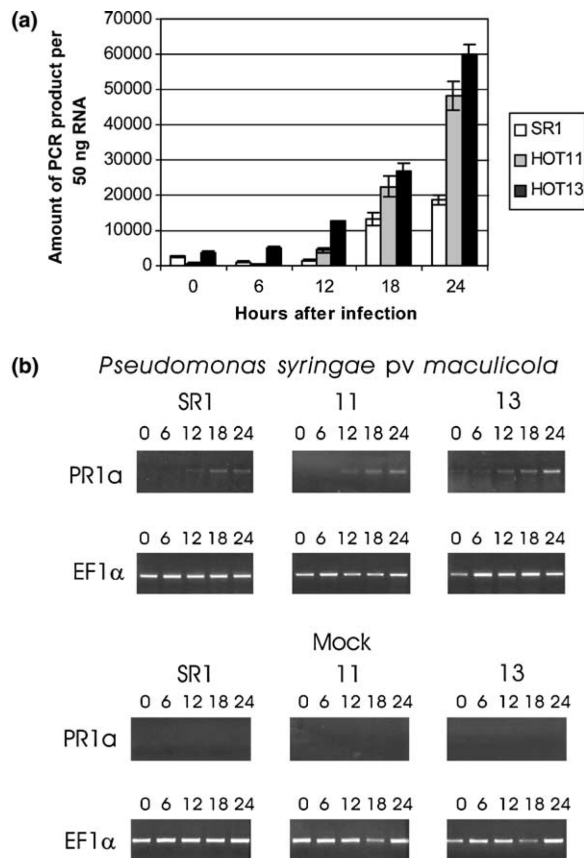

Fig. 4. (a) Quantitation of change in *PR-1a* expression levels by real time PCR before and upon infection of tobacco leaves with *P. syringae* pv. *maculicola*. (b) Time course analysis of *PR-1a* gene expression by RT-PCR before and upon infection of tobacco leaves (SR1: non-transformed control; 11, 13: independent HOT lines) with *P. syringae* pv. *maculicola* (0, 6, 12, 18, and 24 h after infection, respectively). To verify the equal loading of RNA samples, the same reaction was carried out using specific primers of *EF1α* gene.

4. Discussion

Recombinant alfalfa class 1 hemoglobin was purified close to homogeneity and analyzed for its NO-scavenging characteristics. Tobacco lines overexpressing alfalfa class 1

hemoglobin (HOT lines, [5]) were used to study possible consequences of hemoglobin-NO interaction with respect to responses to pathogen infection.

There is an inverse relationship between the levels of class 1 hemoglobins and NO in tissue during hypoxic stress [6,32] and there are implications of a relationship between the two compounds in reducing effects of NO or pathogen attack in HOT tobacco plants [5]. Extracts of alfalfa roots overexpressing barley hemoglobin possess an NAD(P)H-dependent NO scavenging activity that is dependent on the presence of the hemoglobin [7]. The demonstration that partially purified preparations of rMhb1 possess an NAD(P)H- and FAD-dependent NO-scavenging activity indicates that alfalfa class 1 hemoglobin is also capable of participating in NO turnover. As with the case for barley hemoglobin, the high NO-scavenging activity at decreasing hemoglobin concentrations after the Phenyl Superose step (Fig. 2), and the loss of specific activity after the size-exclusion (Superose 12) step indicate that some other protein likely possessing methemoglobin reductase activity is a necessary component in NO-scavenging together with the alfalfa recombinant hemoglobin.

In the case of *Vitreoscilla* hemoglobin (*Vhb*), which is capable of acting either as a single domain homodimer or as a two-domain heterodimer in vivo, NO-scavenging ability was reported to improve significantly upon its association with the flavoreductase domain of *Ralstonia eutropha* flavohemoglobin [33]. However, unlike flavohemoglobins, class 1 hemoglobins do not possess a flavin domain. Furthermore, a loss of bound FAD was observed during flavohemoglobin purification, so external FAD was required to recover the NO-scavenging activity [26]. This may be similar to the observation that addition of external FAD was necessary during purification of the recombinant Mhb1 protein probably to preserve the methemoglobin reductase activity of the above mentioned hypothetical flavoprotein. There are several reports of enzymes that could be potential candidates to reduce methemoglobin in *E. coli* extracts. Studies with *Vitreoscilla* Hb [34,35] have demonstrated that a non-membranous cytochrome reductase, possessing broad specificity to reduce oxidized hemoproteins, copurifies with Vitreoscilla Hb. We have also shown that a pig heart lipoamide dehydrogenase, which also possesses cytochrome reductase activity, can reduce methemoglobin [36].

Similarly to mammalian systems [8,9], a functional hemoglobin-NO interaction *in planta* may also reduce intracellular NO levels and affect a variety of NO-related processes (reviewed in [37–39]). *P. syringae* pv. *glycinea* is known to induce NO-production upon infection [40]. In the case of pathogen infection, the supposed lower levels of NO in Mhb1-overproducing plants could cause a fundamental change throughout the NO-mediated signalling cascade. A balance between NO and H_2O_2 is thought to be necessary for initiating the process of hypersensitive cell death [41]. In HOT plants, the increased levels of NO resulting from the infection process would be expected to be reduced by the reaction with the over-expressed class 1 hemoglobin, disrupting the hypersensitive cell death response and resulting in a smaller necrotic spot, in line with the observed result [5].

In contrast to programmed cell death, the induction of pathogenesis-related genes is known to be a late defense response. The interrelationships between NO, ROS, SA and late defense-response, such as *PR-1a* gene induction, have been well documented [42]. An NO-dependent and a ROS-dependent pathway, both involving SA, have been proposed in the induction of *PR-s*. NO levels in hypoxic plant tissue have been shown to vary independently with Hb [6,33]. In Psm-infected HOT plants, a significant increase was detected in *PR-1a* gene induction compared to controls (Fig. 4). ROS and SA quantities of Psm-infected HOT lines have been found to be significantly higher compared to an uninfected control [5]. Some points with respect to Hb, NO and ROS are germane to the discussion. Both barley [7] and alfalfa hemoglobin (Table 3) are components of an NO-scavenging activity. These cumulative data would suggest that the *PR-1a* response observed in HOT lines occurs via a ROS-SA pathway, since the steady state NO levels in infected plants would be expected to be low due to the presence of hemoglobin.

The present results demonstrate that modulation of NO levels by class 1 hemoglobins have an effect on the HR. Whether these hemoglobins normally participate in the HR is still to be demonstrated. There are, however, results indicating a downregulation of non-symbiotic hemoglobin in *Lotus japonicus* roots upon colonization by its symbiont, the mycorrhizal fungus *Glomus sp* [43]. A NO-scavenging activity was not only observed with rMhb1 protein from alfalfa, but also with barley hemoglobin [7], we can assume that these proteins from both monocot and dicot plants may have such a role. Indeed, among other globins and heme proteins, class 1 hemoglobins of different plants share the same hexacoordinated heme structure [13,14] that results in tight ligand binding [17,44].

In summary, our results implicate class 1 hemoglobin in NO-scavenging and that this scavenging can affect an NO-regulated process such as the HR associated with pathogen infection.

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