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# Phytoglobins can interfere with nitric oxide functions during plant growth and pathogenic responses: a transgenic approach

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**Abstract**

To investigate the possible role of the non-symbiotic plant hemoglobins (phytoglobins) in relation to nitric oxide (NO) functions and their presumable involvement in NO- or pathogenesis-induced necrosis, we have produced transgenic tobacco plants (HOT lines) overexpressing an alfalfa hemoglobin cDNA (*Mhb1*) under the control of CaMV35S promoter. Upon treatment with active sodium nitroprusside (SNP), a widely used NO donor, the germination of seeds and development of seedlings were significantly less retarded in transgenic lines compared with the retardation of non-transformed seedlings. SNP-injection necrotized mature plant leaves of *Mhb1*-transformants to a lower extent than control leaves. Furthermore, infection of tobacco leaves either with *Pseudomonas syringae* pv. *phaseolicola* or Tobacco Necrosis Virus (TNV) resulted in reduced necrosis of mature transgenic plants. In response to bacterial infection, reactive oxygen species (ROS) and salicylic acid (SA) were produced at a higher level in transgenic HOT plants than in control ones. The presented experimental data support a conclusion that plant non-symbiotic hemoglobins are active functional partners in NO-dependent physiological responses such as alteration of plant growth and development as well as cell death and symptom generation after pathogen infection. The described experiments provide new insights to the role of phytoglobins in ROS-, NO- and SA-mediated cellular events during the induction of necrotic cell death.


*Keywords:* Plant non-symbiotic hemoglobin; Nitric oxide; Germination; Cell death; Salicylic acid; Reactive oxygen species

## 1. Introduction

In contrast to the extensive studies on symbiotic hemoglobins, the non-symbiotic hemoglobins termed also as 'phytoglobins' have attracted significant interest only recently (see review by Ref. [1]). Hemoglobins of plant origin represent a multifunctional protein family with diverse structural features [1,2]. The successful cloning of phytoglobin genes from a variety of plant species [2–4] allowed to establish structural characteristics of these proteins and their phylogenetic relations [5]. The corresponding genes are expressed in tissue cultures and during various metabolic stresses such as hypoxia, cold stress and exposure to $NO_3^-$ ions [4,6–8].

The interplay between hemoglobins and nitric oxide (NO) has been shown by studies in mammals and microbes [9,10] and this reaction is considered as a key mechanism in detoxification of NO [11]. This function was also studied in plants. Production of NO was observed during the first 24 h of hypoxia in maize cells. Transformed maize cell lines expressing reduced amounts of phytoglobin produced more NO than wild type or phytoglobin-overexpressing lines [1]. The authors attributed a detoxifying function to phytoglobins during hypoxia by binding NO.

As a signaling molecule, NO could evoke light responses such as stimulation of seed germination, deetiolation and inhibition of hypocotyl elongation on

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lettuce and *Arabidopsis thaliana* seedlings [12]. NO was also reported to inhibit respiration after imbibition of soybean seeds and participate in the control of root growth, maturation and senescence [13–15]. Cell death was shown to be induced by NO both in *Taxus* callus cultures [16] and in *A. thaliana* suspension cultures [17]. Furthermore, NO, similarly to its activatory role in mammalian defense responses [18,19], was found to be a key component of the plant resistance to the infection [20–22]. Divergent functions of NO in different plant cell types include the synergetic interaction with $H_2O_2$ in induction of cell death, the iron mobilization and elevation of oxidative damage or the delay of cell death as antioxidant [21,23,24].

Recently, we have identified a novel phytoglobin cDNA (*Mhb1*, GenBank accession number: AF172172, [7]) that opened opportunities to establish an in vivo experimental system to study the consequences of Mhb1 protein overproduction in NO and immune responses of tobacco plants against pathogens. This approach may have relevance because of the limitations in adapting of methodologies to quantify NO in plant cells and monitor the NO binding to various molecules including hemoglobins that were developed for blood samples [25]. In this paper we compare the responses of transgenic and control tobacco plants to sodium nitroprusside (SNP) treatment. SNP is widely used as NO-generating compound also in plants [16,17,20,24]. The abundant production of phytoglobin prevented mature plant leaves from SNP-induced necrosis and reduced the effects of SNP-caused retardation in growth during germination under light. In addition, leaves of mature transgenic plant infected either with *Pseudomonas syringae* pv. *phaseolicola* or Tobacco Necrosis Virus (TNV) showed reduced symptoms of necrosis as compared to control leaves. Hemoglobin accumulation was accompanied with increased reactive oxygen species (ROS) and salicylic acid (SA) production in the tobacco plant after bacterial infection. The presented in vivo data focus the attention to phytoglobins as functional components in NO- or pathogen-generated plant responses.

## 2. Materials and methods

### 2.1. 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 viral CaMV 35S promoter [26]. 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 Ref. [27]. Expression of the *Mhb1* gene in tobacco plants was verified by Western analysis as described earlier [7].

### 2.2. Seed germination and SNP-treatment of seeds

Seeds derived from the 3rd generation of the *Mhb1*-transformant tobacco plants and seeds of non-transformant SR1 plants were placed on filter paper moisturized with 3 ml of water. Petri dishes were closed and placed to 23 °C in the light for 48 h, then 1 ml of SNP (SIGMA) was added from a 1.2 mM freshly prepared stock solution. The final SNP concentration was approximately 300 μM. To untreated 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 1 day or to artificial light for 2–3 days. The obtained inactive SNP was then used similarly to the above described protocol. Eight-day-old seedlings (on the 6th day of the treatment) were photographed with an Olympus Camedia C2020Z digital camera (Olympus Optical Co. Ltd., Tokyo, Japan) attached to an Olympus SZX-9 (Olympus Optical Co. Ltd., Tokyo, Japan) stereomicroscope. The experiment was repeated four times.

### 2.3. Treatment of tobacco leaves with NO, bacterial suspension or virus inoculum

Seeds of non-transformed tobacco (*N. tabacum* cv. Petit Havanna line SR1) and seeds from the 3rd generation of the *Mhb1*-transformant tobacco (HOT) plants were sown in soil and grown under normal greenhouse conditions (18–23 °C; supplementary light: 160 μE $m^{-2}s^{-1}$ for 8 h $day^{-1}$; relative humidity: 75–80%). For each experiment, 50–60-day-old plants and the 3rd and 4th true leaves (i.e. the 3rd and 4th leaf position above hypocotyl) were used for treatment or inoculation with pathogen. To study the effect of NO on leaf tissues, SNP solutions of various concentrations were injected into the interveinal areas of leaves of control (SR1) and transformed (HOT1, HOT11, HOT13) tobacco lines. Light-inactivated (see above, at least for 24 h before use) and active SNP solutions were injected 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. *P. syringae* pv. *phaseolicola* was maintained on nutrient agar and stored at 4 °C. Bacteria for inoculation were cultured on King's B medium at 25 °C for 24 h, collected in sterile distilled water (SDW) and centrifuged at $1500 \times g$ for 10 min. The bacterial pellet was resuspended in SDW and the concentration adjusted

to $3 \times 10^8$ bacteria $ml^{-1}$. 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.

The 3rd and 4th leaves of control and transformed tobacco plants were inoculated with a suspension of TNV. The virus was maintained in tobacco (*N. tabacum*. cv. Samsun) plants. Leaves of plants showing typical disease symptoms of TNV were ground in a mortar (1 g in 3 ml 10 mM Na-phosphate buffer, pH 7.0), and the homogenate was used for inoculation of tobacco leaves. Lesions on the infected leaves were counted 4–5 days after inoculation.

### 2.4. Measurement of ROS production

ROS concentrations were measured on 10 leaf discs obtained from fully developed leaves of healthy plants or at different timepoints after pathogen treatment.

ROS production was assayed spectrophotometrically according to Doke's method [28] by monitoring the reduction of Nitroblue Tetrazolium (NBT from SIGMA) at 580 nm after incubation with leaf discs punched out from appropriate plants. Data represent one out of three independent experiments. Inoculations were performed on three plants during each experiment.

### 2.5. Quantification of SA and SAG

Two-month-old plants were injected with *P. syringae* pv. *maculicola* at a concentration of $10^8$ cfu $ml^{-1}$. Inoculations were performed on three 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 *quantified* essentially as described by Hennig et al. [29] and Malamy et al. [30]. HPLC was performed as described [31] 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.

## 3. Results

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

Using the *Agrobacterium* transformation system, we have produced several independent transgenic tobacco lines constitutively overexpressing the alfalfa non-symbiotic hemoglobin Mhb1. The presence of the functional *Mhb1* transgene was confirmed by Western blot analysis based on the polyclonal antibody raised against a recombinant Mhb1 protein [7]. Fig. 1 shows that

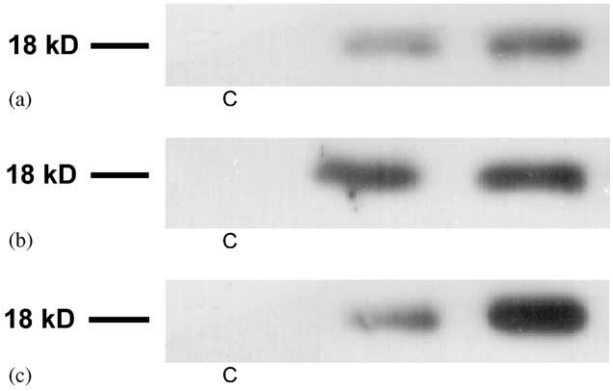


Fig. 1. The immunoblots show the abundant accumulation of the Mhb1 protein in two kamamycin resistant plants from three independent transformed tobacco lines: (a) HOT1; (b) HOT11; (c) HOT13. The polyclonal antibody raised against recombinant Mhb1 protein specifically recognized the alfalfa protein with the expected size of 18 kDa. C stands for negative control (non-transformant SR1 plant).

seedlings of the 3rd generation plants from selected lines (HOT1, HOT11, HOT13) synthesized considerable amounts of the alfalfa hemoglobin. These transformed plants did not exhibit any obvious phenotypic differences in comparison to the control plants grown in the greenhouse.

### 3.2. Reduced sensitivity of hemoglobin-overproducing tobacco HOT seedlings towards SNP as NO-generating chemical

Similarly to mature plants, no obvious phenotypic or growth rate difference could be seen between untreated transformed (Fig. 2Aa) and non-transformed (Fig. 2Ab) seedlings or after treatment with 20 μM SNP (data not shown).

However, a considerable retardation of germination was observed both in the transformed and non-transformed seedlings grown in the presence of a high concentration of NO donor (300 μM SNP) (Fig. 2Ac and Ad, respectively) as compared to the untreated ones (Fig. 2Aa and Ab). The inhibitory effects were recognizable in delayed organ formation and reduced growth as reflected by fresh weight of seedlings (Table 1). As demonstrated by photos in Fig. 2A, the growth and development of transformed seedlings were less retarded by SNP-treatment than their non-transformed counterparts. The Mhb1-overproducing seedlings developed cotyledons and radicles while the non-transformed seedlings formed only radicles (Fig. 2Ac and Ad) during the first 8 days of germination. It is clearly visible that the seedlings treated with 300 μM SNP did not die, but continued germination at a slower speed.

Since SNP is a light-sensitive chemical compound, continuous illumination causes the decomposition of SNP while NO is released. The byproducts of SNP-

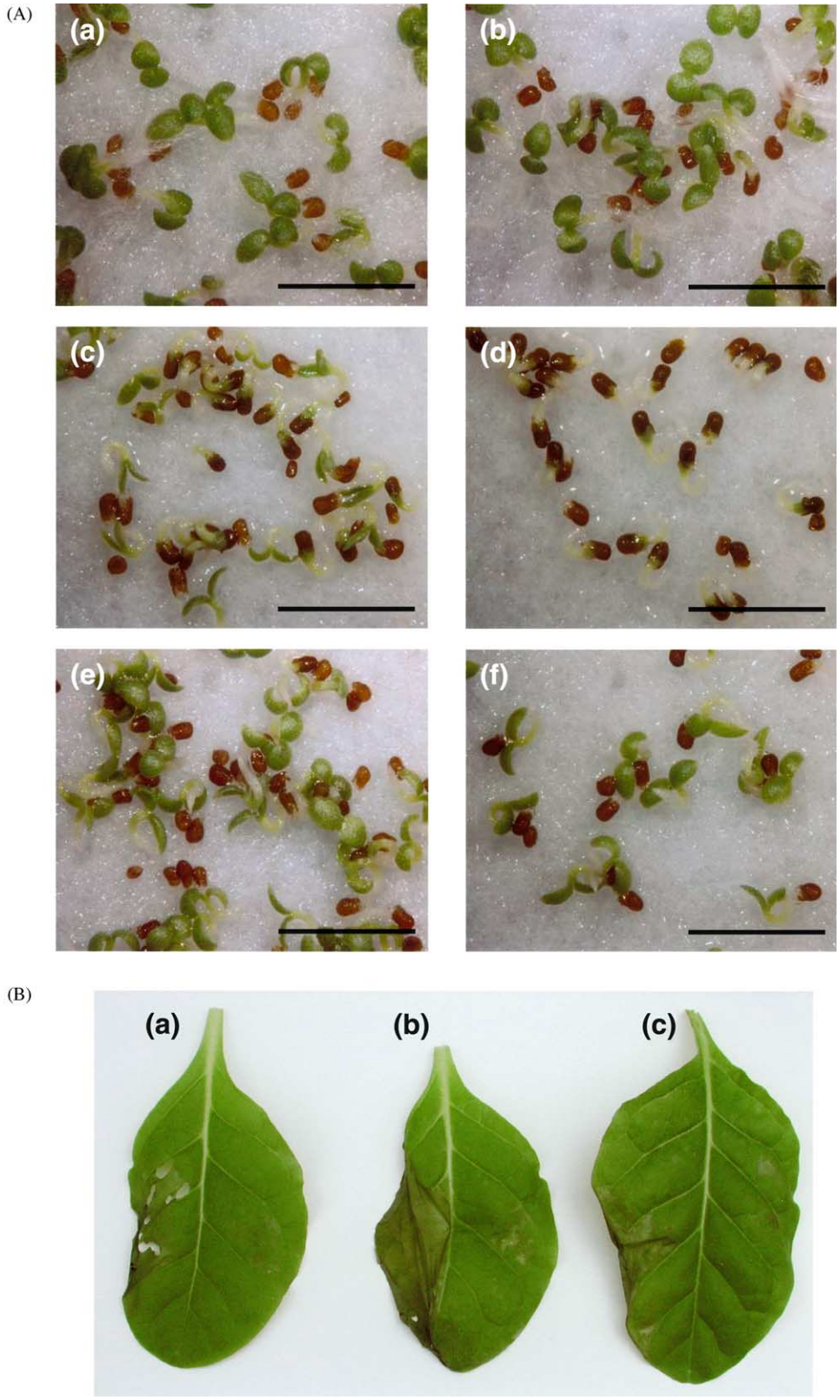


Fig. 2. The hemoglobin overproducing tobacco (HOT) plants are less sensitive to NO-generated responses after treatment with SNP. (A) Photographs of 8-day-old *Mhb1*-transformants (a, c, e) and non-transformed (b, d, f) tobacco seedlings. Untreated seedlings: (a, b); seedlings grown on 300 μM of active SNP: (c, d); seedlings grown on 300 μM of light-inactivated SNP: (e, f). Bars represent 5 mm. (B) Necrotic damages caused by 5 mM SNP solutions 3 days after injection into the leaves of transgenic HOT1 (a), HOT11 (c) and control SR1 (b) tobacco plants. The left half of leaves were injected with active SNP solution, the right half of the leaves were treated with light-inactivated SNP.

Table 1
The seedlings of Mhb1-overexpressing transgenic tobacco HOT lines show reduced sensitivity towards active SNP (300 μM)

| Tobacco lines | Fresh weight of 50 seedlings (mg) upon treatment with active SNP | Fresh weight of 50 seedlings (mg) upon treatment with inactive SNP | Ratio (%) |
|---|---|---|---|
| SR1 | 11.33±2.29 | 17.77±3.38 | 63.75 |
| HOT1 | 18.33±2.31 | 22.00±1.00 | 83.33 |
| HOT11 | 13.33±0.57 | 13.33±0.57 | 100.00 |
| HOT13 | 14.00±1.00 | 18.66±1.15 | 75.00 |

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 light-inactivated SNP, a retardation was observed regardless of the genotype (Fig. 2Ae and Af). However, the non-transformed seedlings treated with active SNP were significantly smaller than those treated with the inactivated SNP (Fig. 2Ad and Af and Table 1). The effect of NO with regard to seedling development is also shown by the fresh weight ratio of seedlings treated with the active and inactivated forms of SNP (Table 1).

### 3.3. *Mhb1-overexpressing plants show tolerance to SNP-induced leaf necrosis*

Based on several experimental data demonstrating the role of NO production in induction of cell death, we have tested necrotization of tobacco leaves from control plants (SR1) and hemoglobin overproducing transformants (HOT1, HOT11, HOT13). Injection of SNP solution into leaf tissues caused concentration-dependent necrotic symptoms. As shown by Table 2A, SNP generated cell death in control leaves already at the concentration of 0.5 mM. At all tested doses of NO donor, the transgenic lines showed reduction in damaged leaf area. Fig. 2B demonstrates the extension of cell death and spread of symptoms to non-treated leaf regions after application of high (5 mM) concentration of SNP. Table 2B presents quantitative data on damaged leaf area by comparing the effects of active and inactive SNP (5 mM). In contrast to actively dividing cells of seedlings, the differentiated cells of mature leaves were only weakly damaged even at this high concentration by the inactive compound. The protective function of elevated synthesis of alfalfa hemoglobin is clearly shown by the data presented.

Table 2
Reduced necrotization of leaves by active SNP treatment in transformed tobacco plants overexpressing the alfalfa non-symbiotic hemoglobin gene.

(A) Damaged leaf area ($mm^2$) after injection of various concentrations of SNP solutions into the leaves of control (SR1) and transgenic (HOT1, HOT11, HOT13) lines

| Tobacco lines | SNP (mM) | | | |
|---|---|---|---|---|
| | 5 | 2.5 | 1 | 0.5 |
| SR1 | 576±128[a] | 480±65[a] | 224±62[b] | 129±101[b] |
| HOT1 | 220±121[b] | 96±122[b] | 0[c] | 0[c] |
| HOT11 | 251±104[b] | 190±73[b] | 0[c] | 0[c] |
| HOT13 | 223±63[b] | 64±72[b] | 0[c] | 0[c] |

(B) Damaged leaf area ($mm^2$). Leaves from different genotypes with 5 mM inactive or active SNP

| Tobacco lines | Inactive SNP | Percentage of damage relative to SR1 leaf area damaged by active SNP (%) | Active SNP | Percentage of damage relative to SR1 leaf area damaged by active SNP (%) |
|---|---|---|---|---|
| SR1 | 53±30 | 9.6 | 549±232 | 100 |
| HOT1 | 27±30 | 4.9 | 199±99 | 36.2 |
| HOT11 | 23±18 | 4.2 | 262±144 | 47.7 |
| HOT13 | 83±67 | 15.1 | 316±159 | 57.5 |

(A) Data with different index letters (a, b, c) are significantly different from each other at $P = 5\%$ level. Data are an average of a representative experiment from two independent experiments with six replicates. (B) Data are an average of four independent experiments with at least four replicates.

### 3.4. Tobacco plants overproducing Mhb1 protein show reduced hypersensitive necrotization after inoculation with P. syringae pv. phaseolicola or TNV

Seeing the differences between control and transformed tobacco lines in response to NO donor SNP, we postulated altered symptoms on hemoglobin overproducing tobacco plants. To test this, we injected incompatible bacteria *P. syringae* pv. *phaseolicola* at various concentrations into transformed and control plant leaves to analyze the extent of damage resulting from hypersensitive necrotization. This incompatible interaction causes hypersensitive response in the non-host tobacco plants. Table 3 shows that the necrotization was suppressed in leaves of transformed plants as compared to control leaves after injection with this bacterium in various concentrations.

Similarly, when transgenic and control plant leaves were infected with TNV, transgenic tobacco lines showed a significant suppression of the lesion number in comparison with control as can be seen in Table 4.

### 3.5. Mhb1-overexpressing plants produce elevated levels of ROS and SA than control plants upon bacterial infection

The detection of alterations in necrotic symptom development in HOT plants encouraged the analysis of changes in the ROS and SA levels of transgenic and SR1 plants upon pathogen attack. We infected tobacco plants with *P. syringae* pv. *maculicola* suspension in concentration $10^8$ ml$^{-1}$. Fig. 3 presents the levels of ROS levels before and after the inoculation of bacteria. In leaves from two transgenic lines (HOT11 and HOT13) elevated ROS concentrations were detected at the time of infection as compared to the control plants. During pathogenic response the transformants produced three to four times more ROS than control SR1 plants.

Table 3
Effect of *P. syringae* pv. *phaseolicola* bacterium suspension when injected into leaves of control (SR1) and transgenic tobacco (HOT1, HOT11, HOT13) lines

| Tobacco lines | Lesion size (cm$^{-3}$) | | |
|---|---|---|---|
| | $2 \times 10^8$ bacteria | $10^8$ bacteria | $5 \times 10^7$ bacteria |
| SR1 | $4.00 \pm 0.00^a$ | $3.83 \pm 0.40^a$ | $1.10 \pm 0.87^b$ |
| HOT1 | $2.33 \pm 0.51^b$ | $1.50 \pm 0.54^b$ | $0^c$ |
| HOT11 | $3.00 \pm 0.89^b$ | $1.80 \pm 0.78^b$ | $0.25 \pm 0.70^c$ |
| HOT13 | $3.66 \pm 0.51^a$ | $1.55 \pm 0.72^b$ | $0.125 \pm 0.35^c$ |

Data with different letters (a, b, c) are significantly different from each other at $P = 5\%$ level. Data are the average of three independent experiments with at least six replicates. Damage scale: 0 = no symptoms; 1 = only chlorosis; 2 = only a small (2–3 mm in diameter) necrotised area; 3 = diffuse necrotisation of the injected area; 4 = the whole injected area is necrotised.

Table 4
The number of lesions on leaves of control (SR1) and transgenic (HOT1, HOT11, HOT13) lines after infection with TNV

| Tobacco lines | Number of necrotic lesions (cm$^{-2}$) | % |
|---|---|---|
| SR1 | $6.80 \pm 0.86^a$ | 100.0 |
| HOT1 | $2.91 \pm 1.11^b$ | 42.8 |
| HOT11 | $2.73 \pm 0.75^b$ | 40.1 |
| HOT13 | $2.07 \pm 0.78^b$ | 30.4 |

Data with different letters (a, b, c) are significantly different from each other at $P = 5\%$ level. Data are the average of three independent experiments with at least six replicates.

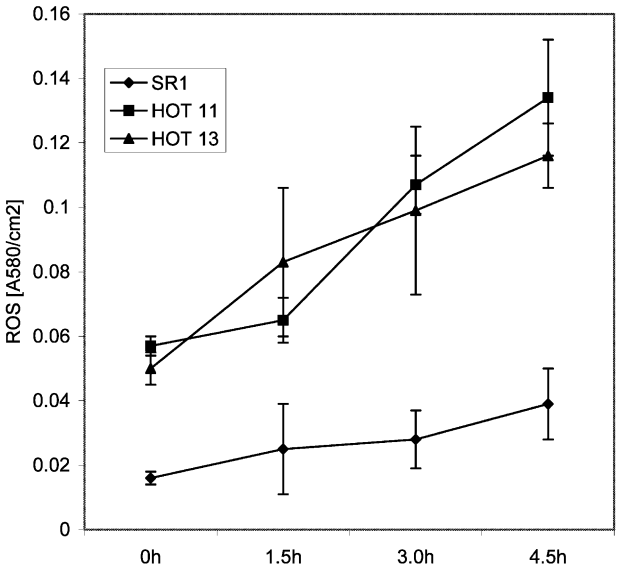


Fig. 3. Differences between transgenic (HOT11, HOT13) and control (SR1) plants in ROS accumulation before and after infection with *Pseudomonas* bacteria (Psm 108).

To characterize the actual status of defense system in these plants we have determined the amounts of free and conjugated SA in the infected leaves. As summarized in Table 5, at 24 h post-injection with *P. syringae* the transgenic HOT plants accumulated three times higher amount of total SA. A slight difference can be seen in the sum of the free and conjugated salicylic acid (SA/SAG) values in the mock inoculated samples. Both the ROS and SA data indicate an enhanced stress response in the hemoglobin overproducing tobacco plants.

## 4. Discussion

In this study, we used phytoglobin-overproducer tobacco (HOT) lines to analyze the physiological role of this protein in NO-related cellular functions with regard to the inhibition of germination, seedling growth and necrotic cell death induced by chemical (SNP) or

Table 5
SA level is higher in transgenic plants overexpressing *Mhb1* in comparison with SR1 after injection with *P. syringae* pv. *maculicola* ($10^8$ bacteria $cm^{-3}$)

| Tobacco lines | SA+SAG concentration ($\mu g\ g^{-1}$ FW) | |
|---|---|---|
| | Mock inoculated (10 mM $MgCl_2$) | *P. syringae* |
| SR1 | 0.96±0.27 | 1.47±0.078 |
| HOT11 | 1.23±0.15 | 4.51±0.03 |
| HOT13 | 1.28±0.14 | 4.68±0.63 |

Free (SA) and conjugated (SAG) salicylic acid levels in the *P. syringae*- or mock-inoculated (10 mM $MgCl_2$) leaves 24 hpi. Results are the mean of two independent experiments. Inoculations were performed on three plants during each experiment. At each time point, SA and SAG levels were determined from the same leaf material. Numbers represent mean values with the standard deviation.

pathogen treatment. The presented data revealed a definite contribution of the alfalfa phytoglobin to the reduction of damages caused by the NO-generating compound (SNP) or infection with viral or bacterial pathogens. Different hemoglobins can ligate oxygen as well as NO [9,10], but the hemoglobin-NO interaction in plant cells has not been shown yet. The presented data indirectly strengthen a postulation that a functional interaction between hemoglobins and NO may have major biological consequences in plant cells, just as it was shown in mammalian cells [25,32]. The transgenic approach based on the altered hemoglobin status of cells can be complementary to biochemical studies, and the present work may encourage further attempts to establish the methodology to monitor hemoglobin-NO interaction in vivo under different conditions. According to our western-blot data, the analyzed HOT lines accumulated more hemoglobin protein at variable quantities, but since this detection method is semi-quantitative and the observed cellular responses are monitored phenotypically, we did not attempt to propose a quantitative correlation. Furthermore, the interplay between hemoglobins and NO may vary according to the actual physiological state of the plants. Indeed, NO itself was shown to generate very divergent responses depending on the plant material. In cultured soybean cells, a low dose of NO caused cell death while higher than 5 mM SNP failed to trigger this pathway [21]. In tobacco leaves the artificial elevation of NO level resulted in significant accumulation of PR-1 protein [22]. Furthermore, in barley aleurone layers NO delayed programmed cell death [24] and NO can act as potent antioxidant during photo-oxidative stress [33].

Although SNP as a NO-releasing compound has already been used in several previous experiments with plants [12,16,17,20], the interpretation of the experimental data should be based on the fact that the present analysis has also clearly revealed some unspecific, inhibitory effects of SNP decomposition byproducts. However, the considerable differences in seedling growth after treatment with active and inactive SNP reflect definite NO effects in SNP-treated cells. The observed inhibition of germination and seedling growth by NO may originate from the variety of alterations in basic cellular functions. The reduction of cell division or the induction of programmed cell death through the activation of a mitogen-activated protein kinase (MAP) was observed in *Arabidopsis* cell suspension culture after SNP-treatment [17]. The role of NO in cell cycle progression is expected to be dependent of the actual concentration of this gas in the cells. In alfalfa protoplast-derived cells more than 30 μM SNP reduced the number of S-phase, but short exposition to low dose of SNP (below 30 μM) could activate the entry into the division cycle [34]. Beligni and Lamattina [12] reported the stimulation of lettuce seed germination by NO donors under dark conditions. Here, we detected the inhibition of growth and development in light grown seedlings. NO was shown to inhibit photosynthetic ATP synthesis [35], therefore, we can expect significant differences in responses under dark or light conditions.

A considerable number of publications support the involvement of NO-signaling in pathogenesis. Inhibitors of NO-synthesis could reduce the hypersensitive reaction of *Arabidopsis* leaves infected by *P. syringae* pv. *maculicola* [20]. Inhibition of NO synthase could decrease the Tobacco Mosaic Virus (TMV)-induced NO production of tobacco leaves [22]. In the case of soybean, NO could act synergistically with $H_2O_2$ [21], with other ROS in *Arabidopsis* leaves to cause cell death [20] and also with SA in the case of tobacco [36]. However, NO could also act independently of ROS to induce the expression of defense-related genes in the case of *Arabidopsis* cell suspension culture [17]. In our work, we followed two approaches in the analysis of NO-related responses after increase of phytoglobin level in tobacco plants. We describe here that injection of SNP into the leaf tissues can generate necrotic symptoms being spread to the non-treated leaf regions. Comparison of the active and inactivated compounds suggests that SNP action is a specific event, so the produced NO is primarily responsible for the symptom development through the induction of cell death. This methodology also enabled us to show that enhanced phytoglobin synthesis 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 a similar response to different necrotrophic pathogens such as *P. syringae* and TNV. On the other hand, the sizes of the lesions caused by TMV were significantly reduced by pretreatment of leaves with NO-releasing compounds [37], which is probably due to an induced (acquired) resistance mechanism.

At this stage of research we describe the protective functions of hemoglobin during necrotization that can rely on different mechanisms. The NO function, the oxidative burst and their combined effects can equally be modified by the availability of hemoglobin in plant cells. The similar differences in responses to both SNP and necrotic pathogens between the control and transformants suggest a key role for hemoglobin–NO interplay in generation of cell death symptoms. In spite of the limitation that presently we cannot provide biochemical proofs for interaction between plant hemoglobins and NO, the described characterization of HOT plants can highlight some key factors in this complex cellular response. If we postulate NO scavenging or breakdown by plant hemoglobins is similar to what has been documented in the case of human hemoglobins [25,38], the synergetic interaction between NO and ROS such as $O_2^-$, $H_2O_2$ (described by Refs. [21,22,33,39]) is expected to be altered in HOT plants. Indeed, we detected significant increase in superoxide anion ($O_2^-$) levels in these transformed plants. The enhanced accumulation of superoxide anion detected by NBT reduction in the infected transformants may indicate a reduced efficiency in peroxynitrite ($ONOO^-$) generation where NO reacts with ROS ($O_2^-$). Based on the model described by Delledonne et al. [21], the HOT plants can represent a physiological state where the NO/$O_2^-$ balance is in favor of $O_2^-$ that can trigger $H_2O_2$-mediated defense reactions. Under limited NO-availability in the cells of HOT plants, the production of singlet oxygen or hydroxyl radical through NO/$H_2O_2$ interaction [40] is also expected to be reduced which can moderate cellular damages observed in these plants. The recent work from Orozco-Cárdenas and Ryan [41] reported that SNP-generated NO reduced $H_2O_2$ production in tomato after elicitor treatment. Moreover, in potato leaves the SNP-treatment did not modify either the amount or the activity of superoxide dismutase (SOD) [33]. If the $H_2O_2$-generating system is not affected by NO, the chemical reactions between ROS and NO—resulting in the generation of peroxynitrite—can be suggested as a basic mechanism that is closely depending on NO availability in the cells. Through this mechanism the overproduction of hemoglobin can directly interfere with NO-dependent ROS levels responsible for the cellular damages.

Considering the iron mobilization functions of NO [42,43], a reduction in biologically active NO can be proposed as the origin of moderate symptom development in SNP-treated or infected leaves from transformed plants. Furthermore, availability of metabolically active iron altered by the changes in NO-levels can also affect chlorophyll content of leaves and the transcript levels of D1 protein of PSII and Rubisco large subunit transcript levels [44].

The essential functions of both NO- and SA-signaling in the activation of plant defense reactions can also rely on the interrelation between these pathways [23]. SA has been reported to act both in the establishment of systemic acquired resistance (SAR) and also in the elaboration of local defense responses in the infected tissue [45]. Production of SA can be both NO-dependent [23] and independent [46]. In the latter case, it depends on ROS. Previously, it was shown that treatment of tobacco leaves with mammalian induced NO synthase (i-NOS) resulted in significant increase in the total SA level [22]. Here, we report a significant accumulation of SA in HOT plants during bacterial infection. This elevated level of SA in the infected transformants can relate to the altered ROS ($O_2^-$) status of the cells shown in this work. The excess $O_2^-$ may be used in $H_2O_2$ generation system and the produced $H_2O_2$ can activate the defense system and pathogen tolerance in tobacco [45]. The presented data showing the reduction of necrotic symptoms in infected tissues of HOT plants with high amounts of $O_2^-$ and SA can alternatively be discussed with the help of results from studies on LSD1 protein function [47,48]. This protein was suggested to sense both $O_2^-$ and SA levels and serve as rheostat by activating the cell death program. Therefore, HOT transgenic plants with amplified $O_2^-$ and SA signals can also offer the possibility of unique experiments to approach several opened questions concerning LSD1 protein function.

Overproduction of SA in tobacco plants enhanced resistance against different pathogens [49].

The present studies also provide a basis to propose that the native phytoglobins synthesized *in planta* either in leaves [50] or in vascular tissues [3] upon pathogen attack can exhibit a protective function similar to what we show in the case of transgenic plants.

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