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PHYTOGLOBINS AND NITRIC OXIDE: NEW PARTNERS IN AN OLD SIGNALLING SYSTEM IN PLANTS

Review

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The recent review summarizes the major achievements in discovery of role of phytoglobins in mediation of nitric oxide generated cellular functions in higher plants. Genes encoding non-symbiotic hemoglobins have been cloned from several plant species. The expression pattern of these genes show tissue-specificity that is also under the control of stress factors like hypoxia. The nitric oxide has pivotal role in signalling pathway specifically in hypersensitive reactions and programmed cell death. Production of transgenic tobacco plants overexpressing the alfalfa hemoglobin showed altered necrotic symptoms after treatment with nitric oxide generating compounds or infection by necrotic pathogens. The present review helps to outline the similar relation between hemoglobin and nitric oxide in plants as it was found in animal cells.

Keywords: Hypoxia – germination – pathogens – salicylic acid – reactive oxygen species

INTRODUCTION

The discovery of plant non-symbiotic hemoglobins, or "phytoglobins" [4, 9], came significantly later than that of symbiotic hemoglobins [31]. Although the function of the symbiotic hemoglobins had long been known [8] by that time, of course the appearance of novel plant hemoglobin types 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 [6]. Inducibility by hypoxia and evidence for barley hemoglobin to take part in ATP metabolism also supported the distinct function of non-symbiotic hemoglobins [51]. Another milestone was to discover the importance of NO-binding by hemoglobins in animals [20]. The investigation of the possible functions of NO

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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 [23, 60]. 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 [22, 38]. The interaction of hemoglobins with NO in a wide range of organisms from unicellulars to animals led to the investigation of such a relation *in planta*. These efforts using transgenic plants overexpressing non-symbiotic hemoglobins have brought remarkable results recently.

General remarks on hemoglobins

Hemoglobins are common to be found in various organisms ranging from unicellulars to higher plants and animals [3, 19, 44, 61]. The hemoglobins of higher organisms are either monomeric (e.g. leghemoglobin, [3]) or multimeric proteins, such as mammalian myoglobin and hemoglobin [61] and some plant non-symbiotic hemoglobins [25].

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.

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 [2].

Non-symbiotic plant hemoglobins, termed also as "phytoglobins" [16], belong to the second group. They are not only present in plants containing symbiotic hemoglobins [1, 2, 4, 12, 34], but also in other plant species such as *Arabidopsis* [56], barley [55], rice [5], *Trema tomentosa* [9], alfalfa [48], etc. Furthermore, symbiotic hemoglobins are assumed to have evolved from non-symbiotic hemoglobins by gene duplication [34]. That is why non-symbiotic hemoglobins are considered not only to be more widespread, but also more ancestral than symbiotic hemoglobins [1]. 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 [16]. Because the group of non-symbiotic hemoglobins has only been discovered relatively recently [4, 9], their function is not yet fully understood.

In general, the members of this group have much higher affinity for oxygen compared to symbiotic hemoglobins [6, 17] and are induced in plants under low oxygen tensions, which attributed an oxygen sensing function to phytoglobins [55, 56].

Hemoglobin genes from various plants (e.g. from *Brassica*, *Gossypium* and *Arabidopsis thaliana*) with close homology to non-symbiotic hemoglobins were shown to be induced by cold stress, not by hypoxia [56]. This gave the basis for the classification of non-symbiotic hemoglobins. According to Trevaskis et al., to class 1 those hemoglobin genes belong that are induced upon hypoxic treatment (e.g. *Mhb1* from alfalfa, *AHB1* from *Arabidopsis thaliana,* a non-symbiotic hemoglobin gene from barley, etc.). The cold-inducible non-symbiotic hemoglobin genes belong to class 2 [56].

Actually, it is not only hypoxia where class 1 genes have a role. For example the class 1 *AHB1* gene of *Arabidopsis thaliana* could also be induced by nitrate [58]. 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 tissues. [42].

Barley non-symbiotic hemoglobin 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 non-transformed 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 [51].

High mRNA levels of phytoglobin were observed in the roots and rosette leaves of barley [55], young leaves, stems and roots of soybean [1], in rice leaves and roots [5], in *Arabidopsis thaliana* roots [56] and in alfalfa roots [48]. The accumulation of phytoglobin mRNA under non-hypoxic conditions is believed to occur because of the high metabolic activity of the above-mentioned tissues [1].

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 [59].

The phylogram in Figure 1 shows the relation of the above-mentioned three major plant hemoglobin types to each other. The group of non-symbiotic hemoglobins is not only separated to class 1 and class 2 subgroups in the phylogram, but also the class 1 group is further divided to class 1 phytoglobins of dicots and those of monocots. This may indicate a functional difference between them in spite of their close homology. Furthermore, the class 2 phytoglobins show a closer relation to symbiotic hemoglobins than to their class 1 counterparts. The 2-on-2 type hemoglobin, AHB3 is also located on the branch of symbiotic hemoglobins, though it seems to be rather distantly related to them. The hemoglobins from *Trema* and *Parasponia* are separated from the major branches. This can be explained by their unique feature that they can fulfil both symbiotic non-symbiotic hemoglobin functions [9].

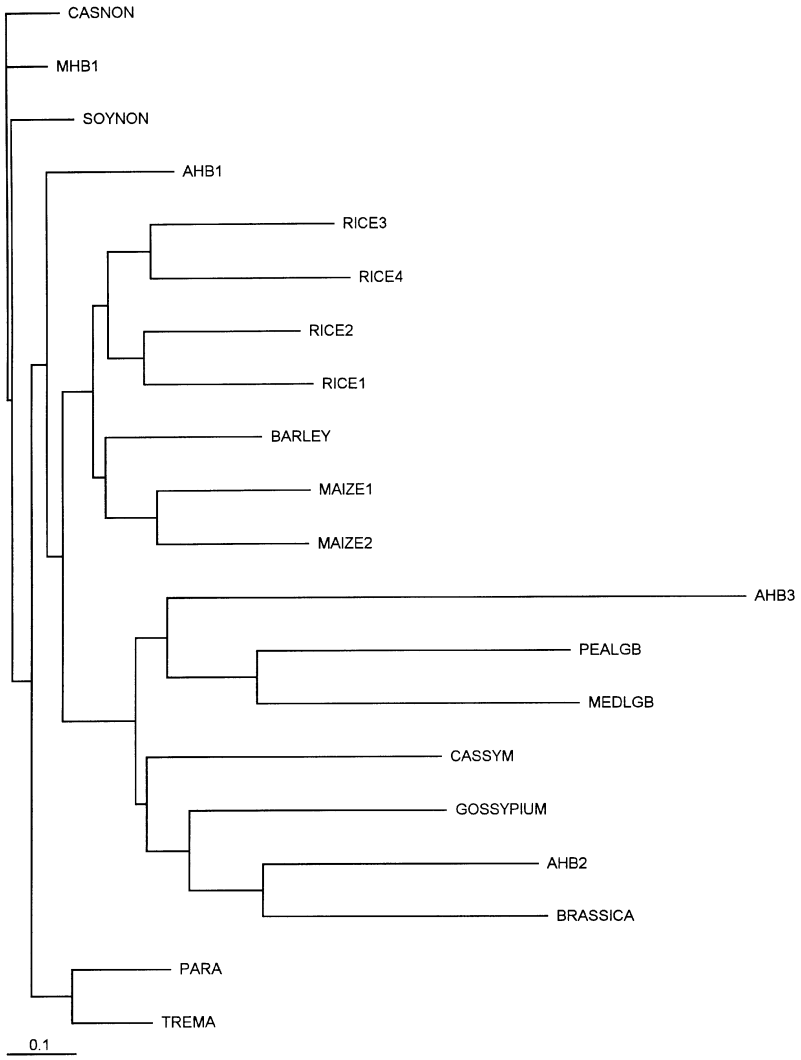

Fig. 1. Phylogram of some plant hemoglobins. Hemoglobins (with GenBank accession numbers or with references): MHB1: (AF172172), CASNON: *Casuarina glauca* non-symbiotic hemoglobin (X53950), SOYNON: soybean non-symbiotic hemoglobin (U47143), TREMA: *Trema tomentosa* non-symbiotic hemoglobin (Y00296), PARA: *Parasponia andersonii* hemoglobin (U27194), AHB1: *Arabidopsis thaliana* class1 non-symbiotic hemoglobin (U94998), MAIZE1 and MAIZE2: maize class 1 non-symbiotic hemoglobins (AAG01375 and AAG01183, respectively), BARLEY: barley non-symbiotic hemoglobin (U01228), RICE1, RICE2, RICE 3 and RICE4: rice class 1 non-symbiotic hemoglobins (U76029, U76028, AAM19124 and AAM19123, respectively), BRASSICA: *Brassica napus* class 2 non-symbiotic hemoglobin (AAK07741), GOSSYPIUM: *Gossypium hirsutum* class 2 non-symbiotic hemoglobin (AAK21604), AHB2: *Arabidopsis thaliana* class 2 non-symbiotic hemoglobin (U94999), CASSYM: *Casuarina glauca* symbiotic hemoglobin [30], MEDLGB: *Medicago sativa* class 1 leg-hemoglobin (X13375), PEALGB: pea leghemoglobin (AB015720)

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 manner. The study of this interaction led 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 [20, 21, 54].

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 [38]. In bacteria, the flavohemoglobins are thought to act as dioxygenases using O_2 to detoxify NO in order to avoid nitrosative stress [22].

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) [40]. 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 [14, 46]. 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 [60].

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. For example in animals, NO can function as a messenger involved in several pathophysiological processes including programmed cell death [37] and those of immune, nervous and vascular systems [47, 57]. The NO signalling in animals can either be cyclic guanosine monophosphate (cGMP)-dependent or- independent [22]. 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 [47].

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 [7]. The situation of peroxynitrite formation is similar in plant defense responses [60], but its importance was shown to be less in the hypersensitive reaction (HR) than in animal cells [15].

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 [52, 53]. One such protein that NO activates by S-nitrosylation is $p21^{ras}$ [53]. This leads to the induction of MAP kinase cascades which can induce apoptosis [33]. NO has been shown to indirectly modify the MAP kinase activity in mammalian tumor cells and neurons [39, 62]. It is important to remark that NO can also inhibit apoptosis [28].

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) [13]. Apoptotic cell death was also shown to be induced by NO in *Taxus* callus cultures [43].

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 [60]. Similarly, the cGMP levels of tobacco increased when cells were treated with NO [18].

NO, similarly to its activatory role in mammalian defense responses [47, 52], is an important component of the plant disease resistance system [14]. 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. Consequently, cGMP and cADPR are second messengers of NO in plants, and they can act synergistically [29], just as reported for gene activation in animal cells [36].

Hypersensitive reaction (HR) is 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 [10] 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 [27,

35]. 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 [11]. Sometimes NO was observed to act synergistically with SA and ROS [29, 52]. 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 [14]. 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 [57]. However, NO can also act independently of ROS to induce the expression of defence-related genes in the case of *Arabidopsis* cell suspension culture [13] and tobacco [18]. 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 results the formation of highly reactive species from the less reactive ones [32, 35]. 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 [24]. 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 [41].

Interaction between phytoglobins and NO in plants

The above results gave basis to assume that the interaction between hemoglobins and NO is possible not only in unicellulars and animals, but also in plants. Recent results seem to support this hypothesis.

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 by transforming it to nitrate (NO_3^-), which is less toxic for plants. 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 [16].

Transgenic tobacco seedlings overexpressing alfalfa phytoglobin Mhb1 [49] were shown to grow less slowly upon treatment with NO-generating compound (SNP) than non-transformed seedlings. Furthermore, adult leaves of Mhb1-expressing plants showed a lower extent of necrosis than non-transformed control after treating with SNP. Both findings are assumed to be the consequence of lower intracellular NO levels caused by NO-phytoglobin interaction.

Infection of adult Mhb1-expressing tobacco with the bacteria *Pseudomonas syringae* (Psm) or with Tobacco Necrosis Virus (TNV) also caused a lower extent of leaf necrosis on transformant plants compared to non-transformed control. In the

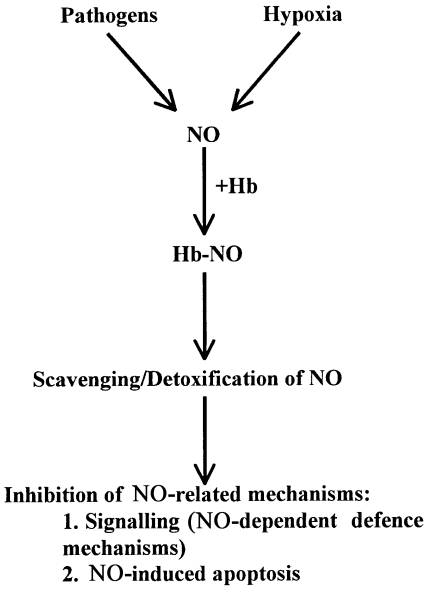

Fig. 2. The possible effect of NO-phytoglobin interaction on NO-dependent mechanisms

transformants, before and after infection with Psm, higher superoxide (O^-) and salicylic acid (SA) levels were measured than in Psm-infected non-transformed plants [49]. It is hypothesized that the lower extent of necrosis on transformant leaves is also the result of intracellular NO level decrease caused by NO-phytoglobin interaction. However, the lower extent of necrosis may not necessarily mean increased resistance of the Mhb1-expressing tobaccos even in spite of their higher ROS and SA levels. This is possible because of the lack of the earlier described synergistic effect [14] between ROS and NO, which cannot be fully compensated by increasing the level of other components in the defence system, e.g. ROS and SA. Figure 2 summarizes how NO-involving processes can be affected by NO-phytoglobin interaction.

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