RESEARCH NOTE

REVISED Effect of the synthesis of rice non-symbiotic hemoglobins 1 and 2 in the recombinant *Escherichia coli* TB1 growth [version 2; referees: 2 approved]

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v2 **First published:** 12 Oct 2015, **4**:1053 (doi: 10.12688/f1000research.7195.1)
Latest published: 01 Mar 2016, **4**:1053 (doi: 10.12688/f1000research.7195.2)

Abstract

Non-symbiotic hemoglobins (nsHbs) are widely distributed in land plants, including rice. These proteins are classified into type 1 (nsHbs-1) and type 2. The O_2-affinity of nsHbs-1 is very high mostly because of an extremely low O_2-dissociation rate constant resulting in that nsHbs-1 apparently do not release O_2 after oxygenation. Thus, it is possible that the *in vivo* function of nsHbs-1 is other than O_2-transport. Based on the properties of multiple Hbs it was proposed that nsHbs-1 could play diverse roles in rice organs, however the *in vivo* activity of rice nsHbs-1 has been poorly analyzed. An *in vivo* analysis for rice nsHbs-1 is essential to elucidate the biological function(s) of these proteins. Rice Hb1 and Hb2 are nsHbs-1 that have been generated in recombinant *Escherichia coli* TB1. The rice Hb1 and Hb2 amino acid sequence, tertiary structure and rate and equilibrium constants for the reaction of O_2 are highly similar. Thus, it is possible that rice Hb1 and Hb2 function similarly *in vivo*. As an initial approach to test this hypothesis we analyzed the effect of the synthesis of rice Hb1 and Hb2 in the recombinant *E. coli* TB1 growth. Effect of the synthesis of the O_2-carrying soybean leghemoglobin *a*, cowpea leghemoglobin II and *Vitreoscilla* Hb in the recombinant *E. coli* TB1 growth was also analyzed as an O_2-carrier control. Our results showed that synthesis of rice Hb1, rice Hb2, soybean Lba, cowpea LbII and *Vitreoscilla* Hb inhibits the recombinant *E. coli* TB1 growth and that growth inhibition was stronger when recombinant *E. coli* TB1 synthesized rice Hb2 than when synthesized rice Hb1. These results suggested that rice Hb1 and Hb2 could function differently *in vivo*.

This article is included in the Oxygen-binding and sensing proteins channel.

Open Peer Review

Referee Status: ✓ ✓

	Invited Referees 1	2
REVISED version 2 published 01 Mar 2016		✓ report
version 1 published 12 Oct 2015	✓ report	? report

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How to cite this article: Álvarez-Salgado E and Arredondo-Peter R. **Effect of the synthesis of rice non-symbiotic hemoglobins 1 and 2 in the recombinant *Escherichia coli* TB1 growth [version 2; referees: 2 approved]** *F1000Research* 2016, **4**:1053 (doi: 10.12688/f1000research.7195.2)

Grant information: This work was partially financed by SEP-PROMEP (grant number UAEMor-PTC-01-01/PTC23) and Consejo Nacional de Ciencia y Tecnología (CoNaCyT grant numbers 25229N and 42873Q), México, to RA-P.
The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: No competing interests were disclosed.

First published: 12 Oct 2015, **4**:1053 (doi: 10.12688/f1000research.7195.1)

REVISED **Amendments from Version 1**

We incorporated some of the references suggested by Dr. Matilla into the revised version of the article and indicated in the legend to Figure 1 that molecular sizes and molecular masses correspond to the Hb cDNAs and proteins analyzed in this work.

See referee reports

Introduction

Non-symbiotic hemoglobins (nsHbs) are O_2-binding proteins widely distributed in land plants, including rice[1]. The nsHbs are classified into type 1 and type 2 (nsHbs-1 and nsHbs-2, respectively) based on sequence similarity and O_2-affinity[2,3]. The O_2-affinity of nsHbs-1 is very high mostly because of an extremely low O_2-dissociation (k_{off}) rate constant[3–5] resulting in that nsHbs-1 apparently do not release O_2 after oxygenation[6,7]. In contrast, the O_2-affinity of nsHbs-2 is moderate mostly because of a moderate to high k_{off} rate constant for O_2, thus apparently nsHbs-2 easily release O_2 after oxygenation[2,3,6,7]. Hence, it is possible that the *in vivo* function of nsHbs-1 is other than O_2-transport and that nsHbs-2 function *in vivo* as O_2-carriers.

Five copies (*hb1* to *5*) of the *nshb* gene have been detected in the rice genome, which are differentially expressed in embryonic and vegetative organs from plants growing under normal and stress conditions[8–11]. Based on the available information on the properties of rice nsHbs and data from the analysis of other plant and non-plant Hbs, it was proposed that rice nsHbs could exhibit a variety of functions *in vivo*, including O_2-transport, O_2-sensing, NO-scavenging and redox-signaling[6,12,13]. However, the *in vivo* activity of rice nsHbs has been poorly analyzed[12]. An *in vivo* analysis for rice nsHbs is essential to elucidate the biological function(s) of these proteins. An approach to analyze the *in vivo* activity of nsHbs is generating knock out rice for individual *nshb* genes, however this is complicated because of the existence of five copies of *nshb* in the rice genome. An alternative approach to analyze the *in vivo* activity of rice nsHbs is examining individual rice nsHbs in a heterologous system, such as recombinant *Escherichia coli*. Rice Hb1[4] and Hb2[14] are nsHbs-1 that have been generated in recombinant *E. coli* TB1. The rice Hb1 and Hb2 amino acid sequence[4], tertiary structure[15] and rate and equilibrium constants for the reaction of O_2[4,14] are highly similar. Thus, it is possible that rice Hb1 and Hb2 function similarly *in vivo*. As an initial approach to test this hypothesis we analyzed the effect of the synthesis of rice Hb1 and Hb2 in the recombinant *E. coli* TB1 growth. Our results showed that synthesis of rice Hb1 and Hb2 inhibited the recombinant *E. coli* TB1 growth and that growth inhibition was stronger when recombinant *E. coli* TB1 synthesized rice Hb2 than when synthesized rice Hb1.

Methods

Untransformed (wild-type) and transformed (recombinant) *E. coli* TB1 (Invitrogen, CA, USA) containing the constitutive pEMBL 18$^+$::Hb1[4], pEMBL18$^+$::Hb2[14], pEMBL18$^+$::Lb*a*[16], pEMBL18$^+$::LbII[17] and pUC18::VHb[18] plasmids were grown in LB broth (Sigma-Aldrich, MO, USA) at 37°C with shaking at 200 rpm. Plasmids pEMBL18$^+$::Lb*a*, pEMBL18$^+$::LbII and pUC18::VHb were included as an O_2-carrier control since they code for the synthesis of the O_2-carrying soybean leghemoglobin *a* (Lb*a*), cowpea leghemoglobin II (LbII)[17,19,20] and *Vitreoscilla* Hb (VHb)[21,22], respectively. The existence of the VHb insert into the pUC18::VHb plasmid was verified by PCR (30 cycles at 55°C/30s for annealing, 72°C/30s for extension and 95°C/30s for denaturation) using specific oligonucleotides (VitHb/ATG: 5´-ATG TTA GAC CAG CAA ACC ATT-3´ and VitHb/TAA: 5´-TTA TTC AAC CGC TTG AGC GTA-3´) designed from the *vhb* sequence deposited in the Genbank database under the accession number X13516. The existence of the Hb1, Hb2, Lb*a* and LbII inserts into the pEMBL18$^+$::Hb1, pEMBL18$^+$::Hb2, pEMBL18$^+$::Lb*a* and pEMBL18$^+$::LbII plasmids, respectively, was verified by *Eco*RI- and *Nco*I (Invitrogen, CA, USA) -double digestion. Inserts were detected by electrophoresis in a 1.4% agarose gel. The existence of recombinant Hbs in cell soluble extracts was verified by SDS-PAGE in a 12.5% polyacrylamide gel. Evaluation of the effect of the Hb synthesis in the recombinant *E. coli* TB1 growth was performed in 50 ml cultures inoculated with ≈5×10^8 colony forming units from a 20 ml overnight culture. Wild-type *E. coli* TB1 was included as control. All assays were performed in triplicate. Cell growth was quantitated by spectrophotometry using $\lambda = 650$ nm for an 8.5 h period.

Results and discussion

Electrophoretic analysis of the PCR reaction and *Eco*RI- and *Nco*I-double digestions showed that plasmids isolated from recombinant *E. coli* TB1 contained inserts corresponding to the rice Hb1[4], rice Hb2[4], soybean Lb*a*[16], cowpea LbII[17] and *Vitreoscilla* Hb[18] cDNAs (Figure 1A). Likewise, analysis by SDS-PAGE showed that rice Hb1, rice Hb2, soybean Lb*a*, cowpea LbII and *Vitreoscilla* Hb existed in the soluble extracts of recombinant *E. coli* TB1 (Figure 1B). This evidence indicated that rice Hb1, rice Hb2, soybean Lb*a*, cowpea LbII and *Vitreoscilla* Hb were synthesized by recombinant *E. coli* TB1.

Figure 2 shows that synthesis of rice Hb1, rice Hb2, soybean Lb*a*, cowpea LbII and *Vitreoscilla* Hb inhibited the recombinant *E. coli* TB1 growth. This was unexpected for soybean Lb*a*, cowpea LbII and *Vitreoscilla* Hb because these proteins would promote cell growth due to their O_2-transport activity[17,19–22]. However, under the conditions tested in this work apparently soybean Lb*a*, cowpea LbII and *Vitreoscilla* Hb affected some aspects of the recombinant *E. coli* TB1 metabolism, possibly owed to the constitutive expression of these proteins into the host cells. Synthesis of rice Hb1 inhibited the recombinant *E. coli* TB1 growth similarly (~37%) to the synthesis of soybean Lb*a*, cowpea LbII and *Vitreoscilla* Hb. This observation suggests that rice Hb1 could function *in vivo* similarly to O_2-carrying Hbs. Likewise, synthesis of rice Hb2 also inhibited the recombinant *E. coli* TB1 growth. However, growth inhibition was stronger (~61%) when recombinant *E. coli* TB1 synthesized rice Hb2 than when synthesized rice Hb1. This observation suggests that rice Hb2 could function *in vivo* by scavenging O_2, possibly owing to its extremely low k_{off} rate constant for O_2[14].

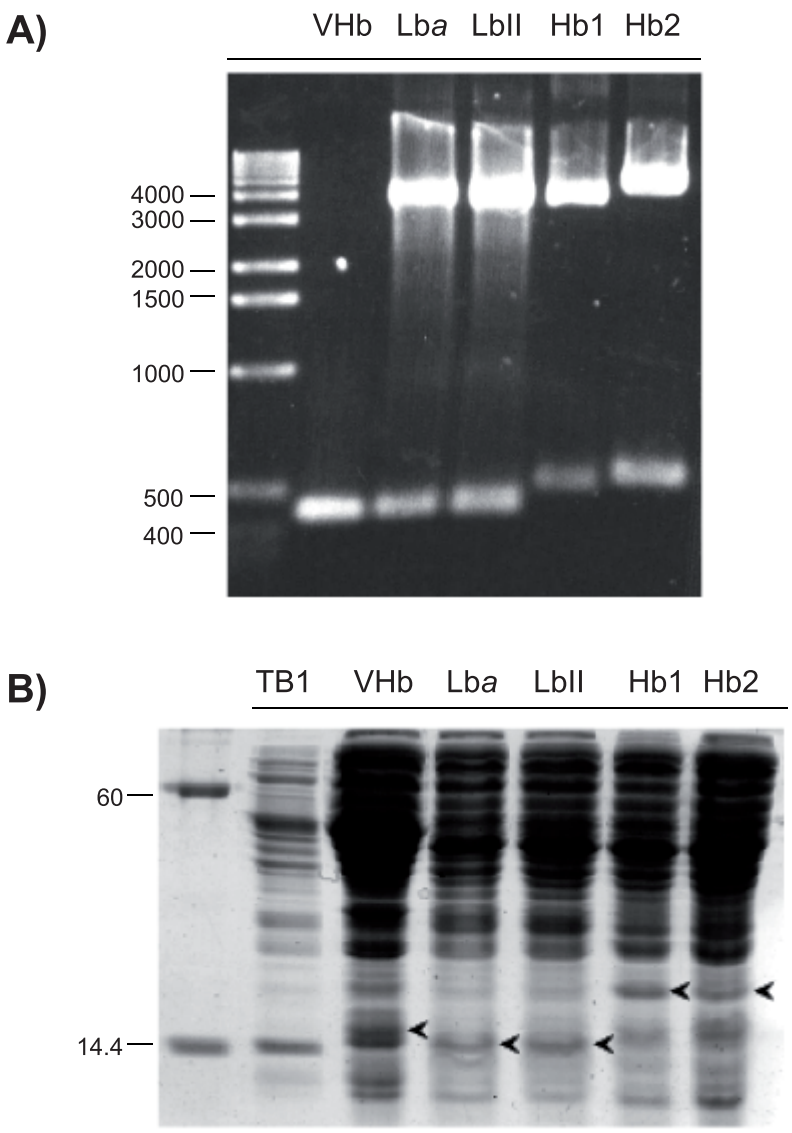

Figure 1. (A) Detection of *Vitreoscilla* Hb PCR fragment and soybean Lb*a*, cowpea LbII, rice Hb1 and rice Hb2 cDNAs from recombinant *E. coli* TB1 by agarose gel electrophoresis. PCR fragment and cDNA sizes are within the 435 to 507 base pairs range, which corresponds to the molecular sizes of the Hb cDNAs analyzed here. Molecular size markers are indicated in base pairs. **(B) Detection of *Vitreoscilla* Hb, soybean Lb*a*, cowpea LbII, rice Hb1 and rice Hb2 proteins (arrow heads) from recombinant *E. coli* TB1 soluble extracts by SDS-PAGE.** A 20 to 50 µg aliquot of total soluble proteins was loaded onto each lane. Recombinant Hb masses are within the 14 to 18.4 KD range, which corresponds to the molecular masses of the Hbs analyzed here. Mass markers are indicated in kD.

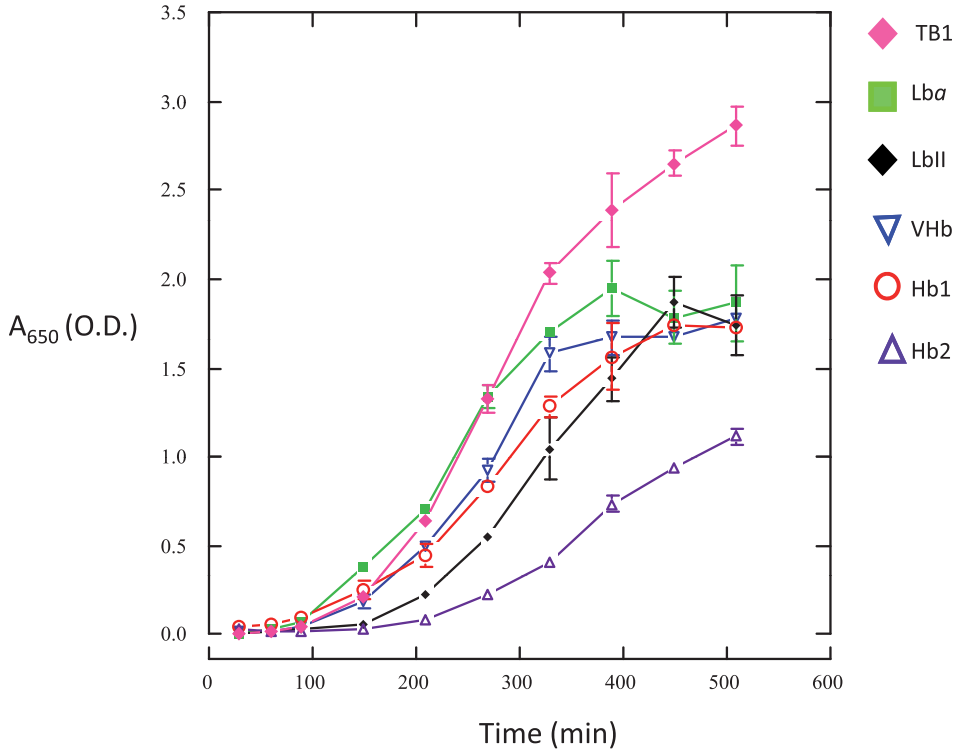

Figure 2. Growth of wild-type (TB1) and recombinant (VHb, Lb*a*, LbII, Hb1 and Hb2) *E. coli*. Values (mean ± SD) correspond to three replicates. See the Methods section for experimental details.

Conclusions

Results presented in this work suggest that in spite of the high similarity between rice Hb1 and Hb2 these proteins could function differently *in vivo*. In order to elucidate the apparent metabolic effects generated by the synthesis of rice Hb1 and Hb2, future work might focus on the physiological and biochemical characterization of recombinant *E. coli* TB1. This may include measuring cell respiratory rates and identifying cell proteins and metabolites using oximetry and proteomic and metabolomic approaches, respectively. Results from these analyses could provide valuable information to understand the *in vivo* function of rice nsHbs.

Author contributions

EAS and RAP conceived the study. EAS executed the experiments. RAP prepared the first draft of the manuscript. EAS and RAP revised the draft manuscript and have agreed to the final content.

Competing interests

No competing interests were disclosed.

Grant information

This work was partially financed by SEP-PROMEP (grant number UAEMor-PTC-01-01/PTC23) and Consejo Nacional de Ciencia y Tecnología (CoNaCyT grant numbers 25229N and 42873Q), México, to RA-P.

I confirm that the funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Acknowledgements

Authors wish to express their gratitude to Dr. Dale A. Webster (Illinois Institute of Technology, USA) for kindly providing the pUC18::VHb plasmid.

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Current Referee Status:  

Version 2

Referee Report 02 March 2016

doi:10.5256/f1000research.8834.r12702

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The authors have sufficiently addressed my concerns.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.

Version 1

Referee Report 23 February 2016

doi:10.5256/f1000research.7751.r12624

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The work by Álvarez-Salgado and Arredondo-Peter (2015) was carefully evaluated. Broadly, this research is worthy of achieving an approval in F1000Research. However, before it achieves this status, is necessary to carry out some minor modifications.

Thus, the first paragraph of the Introduction, referred to the different affinity of nsHbs1 and nsHb2 for O_2, needs to include some recent references (eg. Hoy and Hargrove, 2008; Smagghe et al, 2009; Thiel et al., 2011; among others). Likewise, in the second paragraph of the Introduction (ie. Based on the available information…. NO scavenging and redox-signaling) the following latest references must also be added (ie. Siddiqui et al., 2010; Vigeolas et al., 2010, among others).

With respect to Res & Discuss, (i) the first paragraph should include some reference to show that the bands referred by the authors (Fig. 1A) specifically belong to rice (Hb1, Hb2), soybean (LBA), cowpea (LbII) and Vitreoscilla (Hb) cDNAs. This fact is key in this work. Likewise, bands corresponding to VHb and Lba (Fig. 1B) are confusing to the reader; (ii) I would eliminate from Fig. 2 the results of LBA, LbII and

HBv growth (include as data not shown) because the main importance of this work are the results concerning nb1 and nbII; (iii) "... these proteins would promote cell growth due to their O_2-transport activity"; this conclusion is based in old results and is very risky; this growth promotion should also be referred to higher plants?; please discuss; and (iv) I repeat, some actual references must be also included into discussion.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Competing Interests: No competing interests were disclosed.

Referee Report 06 January 2016

doi:10.5256/f1000research.7751.r11632

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This is an interesting approach to possibly differentiating between the functions of this group of proteins. A fundamental question that arises out of this work is why do hemoglobin proteins inhibit *E. coli* growth? The growth differences between the rice Pgb1.1-transformed line and the rice Pgb1.2 line is particularly interesting. I would be cautious, however, in attempting to interpret the results with respect to the proteins possibly behaving as oxygen carriers/transporters. My concerns are based on the following:

1. Why would a unicellular organism without mitochondria require an oxygen carrier since oxidative phosphorylation occurs on the plasma membrane?

2. If plant phytoglobins have an oxygen carrier function would you not expect the two class 1 phytoglobins to have the same effect since they both have similar oxygen binding characteristics?

3. Why would you anticipate that Pgb1.2 might participate more in NO scavenging than Pgb1.1 in *E. coli* if they both have similar configurations in the heme pocket? Is it not the class 2 Pgbs that are suggested to possibly being less amenable to NO scavenging?

4. Is it possible that the expression of the class 2 protein is interfering with some function of the native flavohemoglobin in E. coli?

5. Although E. coli does not have a true nucleus, is it possible that the protein is specifically interfering with transcriptional/translation functions in the chromosome, e.g., the N-end rule pathway as one possibility?

My other comment concerns the terminology. The individuals who work in this area agreed to forego the use of the term "nonsymbiotic hemoglobins" at an international meeting in 2014, replacing it with "phytoglobin", since the original designation does not appropriately describe the protein. I would hope that the authors consider modifying the manuscript to ensure that the name change becomes recognized in the literature.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.

Author Response 06 Jan 2016

Raul Arredondo-Peter, Universidad Autonoma del Estado de Morelos, Mexico

We thank Dr. Robert Hill for evaluating this article and providing constructive comments and suggestions.

We agree with Dr. Hill´s comments corresponding to questions 1 to 5. Undoubtedly they should also be considered in future research focused to elucidate the physiological effects of the synthesis of rice non-symbiotic hemoglobins 1 and 2 in recombinant *E*. *coli* TB1.

Regarding the terminology, we decided to not change the term “non-symbiotic hemoglobins 1 and 2” by “phytoglobins 1.1 and 1.2” (which was accepted in the 2014 XVIII Oxygen-Binding and Sensing Proteins meeting) because details for the accepted nomenclature have not been published. Thus, the accepted nomenclature is not yet widely available to individuals working/interested in the plant hemoglobins field. Hence, replacing the term non-symbiotic hemoglobins 1 and 2 (which has been used for many years in the literature) by the novel term phytoglobins 1.1 and 1.2 could result as confusing to readers of this article.

Competing Interests: No competing interests were disclosed.No competing interests were disclosed.No competing interests were disclosed.No competing interests were disclosed.