RESEARCH NOTE

REVISED Effect of the synthesis of rice non-symbiotic

hemoglobins 1 and 2 in the recombinant *Escherichia coli* TB1 growth [version 2; peer review: 2 approved]

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v2 First published: 12 Oct 2015, 4:1053
<https://doi.org/10.12688/f1000research.7195.1>
 Latest published: 01 Mar 2016, 4:1053
<https://doi.org/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 O₂-affinity of nsHbs-1 is very high mostly because of an extremely low O₂-dissociation rate constant resulting in that nsHbs-1 apparently do not release O₂ after oxygenation. Thus, it is possible that the *in vivo* function of nsHbs-1 is other than O₂-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 O₂ 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 O₂-carrying soybean leghemoglobin α , cowpea leghemoglobin II and *Vitreoscilla* Hb in the recombinant *E. coli* TB1 growth was also analyzed as an O₂-carrier control. Our results showed that synthesis of rice Hb1, rice Hb2, soybean Lb α , 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*.

Keywords

Function, heterologous expression, *in vivo*, *Oryza*, oxygen

Open Peer Review

Approval Status

	1	2
version 2 (revision) 01 Mar 2016		view
version 1 12 Oct 2015	view	view

1. **Robert Hill**, University of Manitoba, Winnipeg, Canada
2. **Angel Matilla**, University of Santiago de Compostela, Santiago de Compostela, Spain

Any reports and responses or comments on the article can be found at the end of the article.

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

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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.

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

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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; peer review: 2 approved]** F1000Research 2016, 4:1053 <https://doi.org/10.12688/f1000research.7195.2>

First published: 12 Oct 2015, 4:1053 <https://doi.org/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 O₂-binding proteins widely distributed in land plants, including rice¹. The nsHbs are classified into type 1 and type 2 (nsHbs-1 and nsHbs-2, respectively) based on sequence similarity and O₂-affinity^{2,3}. The O₂-affinity of nsHbs-1 is very high mostly because of an extremely low O₂-dissociation (k_{off}) rate constant^{3–5} resulting in that nsHbs-1 apparently do not release O₂ after oxygenation^{6,7}. In contrast, the O₂-affinity of nsHbs-2 is moderate mostly because of a moderate to high k_{off} rate constant for O₂, thus apparently nsHbs-2 easily release O₂ after oxygenation^{2,3,6,7}. Hence, it is possible that the *in vivo* function of nsHbs-1 is other than O₂-transport and that nsHbs-2 function *in vivo* as O₂-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 O₂-transport, O₂-sensing, NO-scavenging and redox-signaling^{6,12,13}. However, the *in vivo* activity of rice nsHbs has been poorly analyzed¹². 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 Hb1⁴ and Hb2¹⁴ are nsHbs-1 that have been generated in recombinant *E. coli* TB1. The rice Hb1 and Hb2 amino acid sequence⁴, tertiary structure¹⁵ and rate and equilibrium constants for the reaction of O₂^{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 pEMBL18⁺::Hb1⁴, pEMBL18⁺::Hb2¹⁴, pEMBL18⁺::Lba¹⁶, pEMBL18⁺::LbII¹⁷ and pUC18::VHb¹⁸ plasmids were grown in LB broth

(Sigma-Aldrich, MO, USA) at 37°C with shaking at 200 rpm. Plasmids pEMBL18⁺::Lba, pEMBL18⁺::LbII and pUC18::VHb were included as an O₂-carrier control since they code for the synthesis of the O₂-carrying soybean leghemoglobin *a* (Lba), 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, Lba and LbII inserts into the pEMBL18⁺::Hb1, pEMBL18⁺::Hb2, pEMBL18⁺::Lba 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 $\approx 5 \times 10^8$ 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 Hb1⁴, rice Hb2¹⁴, soybean Lba¹⁶, cowpea LbII¹⁷ and *Vitreoscilla* Hb¹⁸ cDNAs (Figure 1A). Likewise, analysis by SDS-PAGE showed that rice Hb1, rice Hb2, soybean Lba, 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 Lba, cowpea LbII and *Vitreoscilla* Hb were synthesized by recombinant *E. coli* TB1.

Figure 2 shows that synthesis of rice Hb1, rice Hb2, soybean Lba, cowpea LbII and *Vitreoscilla* Hb inhibited the recombinant *E. coli* TB1 growth. This was unexpected for soybean Lba, cowpea LbII and *Vitreoscilla* Hb because these proteins would promote cell growth due to their O₂-transport activity^{17,19–22}. However, under the conditions tested in this work apparently soybean Lba, 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 Lba, cowpea LbII and *Vitreoscilla* Hb. This observation suggests that rice Hb1 could function *in vivo* similarly to O₂-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 O₂, possibly owing to its extremely low k_{off} rate constant for O₂¹⁴.

Figure 1. (A) Detection of *Vitreoscilla* Hb PCR fragment and soybean Lba, 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 Lba, 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, Lba, LbII, Hb1 and Hb2) *E. coli*. Values (mean $\pm$ 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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<https://doi.org/10.5256/f1000research.8834.r12702>

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The authors have sufficiently addressed my concerns.

Competing Interests: No competing interests were disclosed.

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

Version 1

Reviewer Report 23 February 2016

<https://doi.org/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 O₂, 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 O₂-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.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and 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.

Reviewer Report 06 January 2016

<https://doi.org/10.5256/f1000research.7751.r11632>

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Robert Hill

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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 phyto-globins 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 Pgb's 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.

Competing Interests: No competing interests were disclosed.

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

Author Response 06 Jan 2016

Raul Arredondo-Peter, Instituto de Investigación en Ciencias Básicas y Aplicadas, Universidad Autónoma del Estado de Morelos, Cuernavaca, 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 phytohemoglobins 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.

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