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Dual targeted poplar ferredoxin NADP+ oxidoreductase interacts with hemoglobin 1

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ABSTRACT

Previous reports have connected non-symbiotic and truncated hemoglobins (Hbs) to metabolism of nitric oxide (NO), an important signalling molecule involved in wood formation. We have studied the capability of poplar (*Populus tremula* × *tremuloides*) Hbs PttHb1 and PttTrHb proteins alone or with a flavin-protein reductase to relieve NO cytotoxicity in living cells. Complementation tests in a Hb-deficient, NO-sensitive yeast (*Saccharomyces cerevisiae*) Δ*yhb1* mutant showed that neither PttHb1 nor PttTrHb alone protected cells against NO. To study the ability of Hbs to interact with a reductase, ferredoxin NADP+ oxidoreductase PtthFNR was characterized by sequencing and proteomics. To date, by far the greatest number of the known dual-targeted plant proteins are directed to chloroplasts and mitochondria. We discovered a novel variant of hFNR that lacks the plastid presequence and resides in cytosol. The coexpression of PttHb1 and PtthFNR partially restored NO resistance of the yeast Δ*yhb1* mutant, whereas PttTrHb coexpressed with PtthFNR failed to rescue growth. YFP fusion proteins confirmed the interaction between PttHb1 and PtthFNR in plant cells. The structural modelling results indicate that PttHb1 and PtthFNR are able to interact as NO dioxygenase. This is the first report on dual targeting of central plant enzyme FNR to plastids and cytosol.

1. Introduction

The sequencing of several genomes has shown that the complexity of the proteomes exceeds that of the genomes. One mean of increasing the proteome complexity is the location of the proteins in more than one cell compartment. These dual targeted proteins have been found in various plant species, and they appear to have arisen early in the evolution of land plants [1]. Since the discovery of the first dual targeted plant protein in 1995 [2] the number of characterized dual targeted proteins in plants has grown to over 250 [3]. The dual targeting can be achieved by several ways such as ambiguous targeting signals that direct a protein to two distinct locations, or alternative transcription or translation, where different targeting signals are produced for each locations [4]. The majority of dual-targeted plant proteins known to date

Abbreviations: BCIP/NPT, 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium; BiFC, bimolecular fluorescence complementation; 2-DE, two-dimensional gel electrophoresis; DETA NONOate, (*Z*)-1-[*N*-(2-aminoethyl)-*N*-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate; DIG, digoxigenin; Hbs, hemoglobins; hFNR, heterotrophic ferredoxin NADP+ oxidoreductase; MS, mass spectrometry; NO, nitric oxide; NOD, NO dioxygenation; nsHb, non-symbiotic hemoglobin; ORF, open reading frame; PIPES, piparazine-*N*,*N*′-bis(ethanesulfonic acid); PMF, Peptide Mass Fingerprint; RACE, rapid amplification of cDNA ends; SNP, sodium nitroprusside; trHb, truncated hemoglobin; WT, wild-type.

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are split between chloroplasts and mitochondria, but there are also reports of proteins residing both in *e.g.* chloroplasts and the nucleus, mitochondria and peroxisomes, mitochondria and Golgi as well as chloroplasts and cytosol [3].

Hemoglobins (Hbs) are a versatile set of proteins that reversibly bind oxygen to the iron ion of their prosthetic haem group. Hbs have a crucial role during plant development; without at least one functional non-symbiotic Hb (nsHb), *Arabidopsis thaliana* seedlings die at an early age [5]. Several studies have related nsHbs and truncated Hbs (trHbs) to the metabolism of the signalling molecule nitric oxide (NO). NO has been shown to be involved in multiple important physiological processes in plants, such as the expression of defence genes, xylem cell differentiation, and root development [6,7]. On the other hand, excess NO levels can become toxic to cells by inactivation of critical [4Fe-4S]-containing dehydratases, *e.g.* Krebs cycle aconitase, and inhibition of respiration by binding to the terminal oxidase [8].

Dordas and co-workers were the first to report NO formation in plants during hypoxia and that the NO concentration had an inverse relationship to the level of Hb expression under low oxygen tension [9,10]. Tobacco plants overexpressing the alfalfa *Mhb1* gene were also less prone to necrosis caused by NO-treatment than the controls [11]. The partially purified Mhb1 extracts were later observed to exhibit NO-degradation activity with NAD(P)H as a cofactor *in vitro* [12]. Since then, the modulation of Hb levels has been reported to affect NO emission in several occasions [5,13–16].

Reports with recombinant alfalfa [12] and barley Hb1s [17] have, however, shown that the purification of the proteins to near homogeneity results in the loss of NO scavenging, thus proposing that some other protein component is required for sustaining NO/Hb1 cycle. In accordance with this, NO reactions of rice Hb1, human and *Synechocystis* Hbs have been compared with those of horse heart myoglobin [18]. *In vitro*, all these Hbs were able to destroy NO rapidly, but *in vivo*, only *Synechocystis* Hb was able to replace the NO detoxification capability of *Escherichia coli* flavohemoglobin (flavoHb). Similarly, monohydroascorbate reductase has been shown to facilitate NO scavenging of barley Hb1 only in *in vitro* conditions [17]. Interestingly, the non-symbiotic Hbs legume of *Lotus japonicus* appear mainly in the nuclei *i.e.* the cell compartment without yet known Hb reductases, and it has recently been observed that these nuclear Hbs may be maintained functional by reduced flavins [19].

We have previously shown that ectomycorrhizal fungi increase the expression of both poplar *Hb* genes, *PttHb1* and *PttTrHb*, in *in vitro* grown roots [20]. The expression of *trHb* genes has also been found to increase during root nodule and arbuscular mycorrhiza symbioses [21], as well as after treatments with 1-aminocyclopropane-1-carboxylic acid and polyamines [22]. According to our immunolocalization studies the occurence of PttTrHb overlaps with areas of NO production, *i.e.* the vascular bundles and the site of lateral root formation, suggesting a role in the NO metabolism [23]. The green alga *Chlamydomonas reinhardtii* contains an unusually high number of genes, 12 individual open reading frames (ORFs), encoding TrHbs. Sanz-Luque et al. [24] have recently shown that one of these proteins, THB1, is maintained in its active form by nitrate reductase, and that lower expression of THB1 results in increased nitrate reductase activity. It appears that extent Hb family has diverged during evolution and now encompass several modes of reduction, depending on protein location and/or species.

In the present work, we investigated whether the *Populus tremula* x *tremuloides* PttHb1 and PttTrHb proteins alone or with a flavin-protein reductase are able to relieve NO sensitivity of living cells. The study lead to discovery of novel cytosolic variant of a well-characterized plastid enzyme, ferredoxin NADP$^+$ oxidoreductase FNR, that is able to interact with Hb1 *in vivo*.

2. Materials and methods

2.1. Plant materials and growth conditions

The production of the poplar (*Populus tremula* L. × *tremuloides* Michx.) lines V613 and V617 used in the experiments was described previously [25]. *In vitro* shoots of the lines were proliferated on modified semi-solid MS medium (full strength of $C_{10}H_{12}FeN_2NaO_8$; half stregth of other micro and macro nutrients; 2.22 μM BA and 2.85 μM IAA; sucrose 30 g L^{-1}) [26] under 16:8 h light/dark photoperiod (140–150 μmol m^{-2} s^{-1}) at 24 °C. To induce root formation, shoots were transferred onto growth regulator free medium containing sucrose 15 g L^{-1}.

2.2. Identification of the poplar heterotrophic ferredoxin NADP$^+$ oxidoreductase coding region

Gene homology searches against NCBI database and the whole genome assembly of *Populus trichocarpa* at Phytozome database (http://www.phytozome.net/poplar.php) were performed using *A. thaliana* root-type ferredoxin NADP$^+$ oxidoreductase sequences AI995147 and AI994434 [27]. The PCR primers to amplify the ORFs of poplar heterotrophic ferredoxin NADP$^+$ oxidoreductase *PtthFNR* were designed based on the Phytozome sequence POPTR_0001s38080 and the NCBI EST sequence EF147012.

Total RNA was isolated from roots of the line V617 using KingFisher^{TM}mL Magnetic Particle Processor (Thermo Scientific, Walthman, MA, USA) with the MagExtractor-RNA-Nucleic Acid Purification Kit (TOYOBO, Osaka, Japan) and transcribed into cDNA with Super Script II (Life Technologies, Carlsbad, CA, USA). The coding region of *PtthFNR* was amplified by PCR. The gel-purified amlification product was cloned with a TOPO TA cloning Kit (Life Technologies) and sequenced.

2.3. In situ *hybridization of PtthFNR transcripts*

The coding region of *PtthFNR* gene was amplified from poplar cDNA with primers 5′-TATTCTCGAGATGGCTCATTTGGCTGCACTTTCT-3′ (forward) and 5′-TATTGAGCTCTCAGTACACTTCAACATGCCATTG-3′ (reverse) introducing *Xho*I and *Sac*I sites to the 5′ and 3′ ends of the DNA fragment, respectively (restriction sites underlined). The PCR product was cloned into the pCR®2.1-TOPO vector (Life Technologies) from which it was digested as a *Xho*I-*Sac*I fragment, ligated into pBluescript II SK+ vector (Agilent Technologies, Santa Clara, CA, USA) and linearized for use as a DNA template for the synthesis of riboprobes. Digoxigenin (DIG) labelled sense and antisense probes were synthesized with the (SP6/T7) Dig RNA labelling kit (Roche) using T7 and T3 RNA polymerases as described previously [28].

Leaf samples of line V617 were placed in cold fixative (4% paraformaldehyde in 0.05 PIPES (piperazine-N,N'-bis(ethanesulfonic acid) buffer, pH 7) and incubated in a vacuum for 4 h at room temperature. Subsequently, the samples were kept overnight at +4 °C and embedded into Paraplast plus (Electron Microscopy Sciences, Fort Washington, USA) [29]. Thin sections (10 μm) were cut from the samples using a microtome (Leica Instruments, Wetzlar, Germany) and heat-fixed onto silanized glass slides (Electron Microscopy Sciences, Hatfield, PA, USA). Otherwise sections were treated as described previously [30]. The hybridized DIG labelled RNA probes were detected using anti-DIG conjugated alkaline phosphatase and BCIP/NBT (5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium) reaction as described by Leitch et al. [29]. Slides were mounted in glycerine