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Glutathionyl-hydroquinone reductases from poplar are plastidial proteins that deglutathionylate both reduced and oxidized glutathionylated quinones

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ABSTRACT

Glutathionyl-hydroquinone reductases (GHRs) catalyze the deglutathionylation of quinones via a catalytic cysteine. The two GHR genes in the *Populus trichocarpa* genome, *Pt-GHR1* and *Pt-GHR2*, are primarily expressed in reproductive organs. Both proteins are localized in plastids. More specifically, Pt-GHR2 localizes in nucleoids. At the structural level, Pt-GHR1 adopts a typical GHR fold, with a dimerization interface comparable to that of the bacterial and fungal GHR counterparts. Pt-GHR1 catalyzes the deglutathionylation of both reduced and oxidized glutathionylated quinones, but the enzyme is more catalytically efficient with the reduced forms.

1. Introduction

Recent plant genome analyses identified at least 14 classes of glutathione transferases (GSTs), namely Tau, Phi, Zeta, Theta, Lambda, EF1Bγ, Metaxin, Ure2p, Hemerythrin, Iota, TetraChloroHydroQuinone Dehalogenase (TCHQD), dehydroascorbate reductase (DHAR), microsomal ProstaGlandin E-Synthase type 2 (mPGES-2) and glutathionyl-hydroquinone reductases (GHRs) [1–4]. These GHRs, which are found in bacteria, haloarchaea, fungi and plants, constitute a newly defined GST class due to their ability to reduce glutathionyl-quinones, but not glutathionyl-acetophenone derivatives unlike the closely related Omega GSTs (GSTOs/GTOs) [5–9]. In bacteria, GHRs participate to the reductive dehalogenase pathway as shown for *Sphingobium chlorophenolicum* PcpF whereas the role of *Escherichia coli* YqjG is not clearly established [6,10]. In *Saccharomyces cerevisiae*, the three GHRs were initially referred to as GTO1, GTO2/ECM4 and GTO3 and GTO1 was shown to be involved in sulfur metabolism [11]. In plants, the physiological substrates and functions of GHRs are unknown.

GHRs exhibit a quite conserved CPWA active site sequence, thus belonging to the cysteine-containing GSTs (Cys-GSTs), which also include GSTOs, Lambda and Beta GSTs (GSTLs and GSTBs) and DHARs [12–14]. The presence of the catalytic cysteine is required for deglutathionylation activities [6,8,9,15] as for other Cys-GSTs and glutaredoxins (Grxs) [16,17]. Unlike canonical GSTs, GHRs adopt a particular dimeric quaternary arrangement, defining a new GST structural class named Xi [8,18]. In these proteins, the two monomers associate exclusively via their α-helical domains. Another difference is the presence of a sequence insertion between α2 and β2 and of a unique C-terminal extension. Moreover, the active site contains a conserved tyrosine triad which provides an acid/base catalytic assistance in the reaction mechanism [18].

We report the first characterization of the plant GHR family by studying the transcript level and subcellular localization of the two

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Populus trichocarpa representatives (Pt-GHR1 and Pt-GHR2), and analyzing the enzymatic and structural properties of Pt-GHR1.

2. Materials and methods

2.1. RT-PCR experiments

RNA extraction from eight *P. trichocarpa* tissues and cDNA synthesis were performed as described by Lallement et al. [16]. Transcripts of Pt-GHR1 (Potri.015G121600), Pt-GHR2 (Potri.014G192300) and ubiquitin (Potri.015G013600), used as the reference gene, were amplified by PCR (25, 30 or 35 cycles) using specific primer pairs (Table S1).

2.2. Subcellular localization of GFP fusion proteins

Pt-GHR1 and *Pt-GHR2* coding sequences, with or without the sequences coding for transit peptides, were amplified by PCR from petiole cDNAs with the primers shown in Table S1 and cloned in the pCK-GFP3 vector, upstream and in frame with the GFP coding sequence [19]. The PEND::dsRED construct was used as a control to localize plastidial nucleoids [20]. Young leaves of *Nicotiana benthamiana* were transfected by bombardment as described before [16]. After 18 h, fluorescence of GFP and chlorophyll were observed in leaves respectively at 505–550 nm and beyond 575 nm after excitation at 488 nm on a Zeiss LSM510 confocal microscope. The dsRED fluorescence was excited at 555 nm and observed at 560–615 nm.

2.3. Cloning in bacterial expression vectors and site-directed mutagenesis

Sequences coding for Pt-GHR1 and Pt-GHR2 devoid of the targeting sequences were inserted into pET-28a vector using primers shown in Table S1. For Pt-GHR1, the expressed protein is devoid of the first 34 amino acids. For Pt-GHR2, three constructs deprived of the 54, 57 and 86 N-terminal amino acids were tested. The catalytic cysteine of Pt-GHR1 was substituted by a serine (C49S) or an alanine (C49A) from pET-28a::Pt-GHR1 using mutagenic oligonucleotides (Table S1) and the QuikChange site-directed mutagenesis kit (Agilent Technologies).

2.4. Protein expression and purification

The *E. coli* Rosetta2 (DE3) pLysS strain transformed with pET-28a::Pt-GHR1 wild-type and mutants was grown at 37 °C in LB medium containing kanamycin (50 μg ml^{-1}) and chloramphenicol (34 μg ml^{-1}). At OD_{600nm} of 0.7, 0.5% (v/v) of ethanol was added to the medium and cells were cooled to 4 °C for 3 h. Protein expression was subsequently induced for 16 h at 20 °C with 0.1 mM isopropyl β-D-1-thiogalactopyranoside. Cells were harvested and resuspended in a 30 mM Tris-HCl pH 8.0, 200 mM NaCl buffer. After lysis by sonication, the cell extract was centrifuged (35000×*g*, 25 min, 4 °C). After differential fractionation with ammonium sulfate (40% and 80% saturation), recombinant proteins were purified by size exclusion chromatography (Ultrogel® ACA44, Biosepra) and then by ion exchange chromatography (DEAE-cellulose column, Sigma). The purified proteins were stored in 30 mM Tris-HCl pH 8.0, 200 mM NaCl buffer at −20 °C and concentrations were determined using theoretical molar absorption coefficients of 55997.5 M^{-1} cm^{-1} for Pt-GHR1 and 55935 M^{-1} cm^{-1} for Pt-GHR1 C49S and C49A variants. *Phanerochaete chrysosporium* GHR1 (Pc-GHR1) and *E. coli* YqjG (Ec-YqjG) were obtained as described previously [8].

2.5. Determination of the oligomerization states and molecular masses

Native or reduced GHRs were analyzed either by mass spectrometry as described previously [8] or by size exclusion chromatography onto a calibrated Superdex 200 10/300 column (GE Healthcare). Reduced proteins were obtained by incubation for 1 h with a 10 fold excess of dithiothreitol (DTT) at room temperature.

2.6. Crystallization and structure determination of recombinant Pt-GHR1

Details of crystallization, data collection and structure determination of Pt-GHR1 are described in Supplementary Materials and Methods. Atomic coordinates and structure factors have been deposited in the Protein Data Bank under the accession code 4USS.

2.7. Substrate preparation

Glutathionylated phenylacetophenone (PAP-SG), menadione (MEN-SG), tetralone (TETRA-SG) and quercetin (Q-SG) were prepared as previously described [16,21–23] and analyzed by mass spectrometry. To generate menadiol-SG, 3 mM $NaBH_4$ were added to menadione-SG immediately before enzymatic analyses to avoid rapid re-oxidation into menadione-SG. The substrates (menadione-SG and menadiol-SG) and enzymatic products (menadione and menadiol) were analyzed by LC–MS. Liquid chromatography was performed at 40 °C on a Luna C18 100A column (2 mm × 150 mm, 3 μm, Phenomenex) with a 20 series Shimadzu Prominence UPLC system. The elution was performed with a methanol gradient in water containing 0.1% formic acid. Mass spectrometry detection was carried out with a Shimadzu TQD LC-MS8030 simultaneously in positive and negative ion mode after electrospray ionization.

2.8. Enzymatic activities

Thiol-transferase and DHAR activities were tested using hydroxyethyl disulfide (HED) and dehydroascorbate (DHA) respectively, peroxidase activity using H_2O_2, ter-butyl-hydroperoxide or cumene hydroperoxide, deglutathionylation activity using PAP-SG, TETRA-SG and Q-SG, esterase activity using 5-chloromethyl-fluorescein diacetate (Green CMFDA©, Invitrogen) and glutathionylation activity using 1-chloro-2,4-dinitrobenzene (CDNB), phenethyl-isothiocyanate (phenethyl ITC) and p-nitrophenyl butyrate (PnP butyrate). All these procedures have been described previously [16].

Deglutathionylation of menadione-SG/menadiol-SG was assayed in 30 mM Tris-HCl pH 8.0, 1 mM EDTA buffer by following the appearance of deglutathionylated products by UV/visible spectrophotometry with or without a separation by HPLC. For the assay relying uniquely on spectrophometric measurements, the enzyme and substrate concentrations of the reaction mixture are provided in the figure legends. For the assay involving the HPLC separation of the substrate and the product, the reaction was initiated by adding enzymes, and stopped after 20 s by adding a final concentration of 35% (v/v) ethanol and vigorous mixing. After centrifugation (14000×*g* for 15 min), the reaction products were analyzed by HPLC using a calibrated Gemini 5 μ C18 110A column (3 mm × 150 mm, Phenomenex) equilibrated with water containing 30 mM acetic acid pH 4.16 and 10% acetonitrile at 20 °C by monitoring absorbance at 250 nm. The nature of reaction products was confirmed by LC–MS (see above).