Plant Physiology and Biochemistry 96 (2015) 53–63

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

Molecular cloning, expression profiles and characterization of a glutathione reductase in *Hevea brasiliensis*

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ARTICLE INFO

Article history:
Received 15 February 2015
Received in revised form
21 July 2015
Accepted 21 July 2015
Available online 23 July 2015

Keywords:
Glutathione reductase
Hevea brasiliensis
Expression profiles
Subcellular localization
Oxidative stress
H_2O_2 tolerance

ABSTRACT

Glutathione reductase (GR; EC 1.8.1.7) is an important oxidoreductase that can protect organisms against various oxidative stresses. In this study, a new GR gene, named as *HbGR2*, was isolated from *Hevea brasiliensis*. The *HbGR2* cDNA contained a 1674-bp open reading frame encoding 557 amino acids and the deduced HbGR2 protein showed high identities to the chloroplastic GRs from other plant species. HbGR2 was localized in the chloroplasts of tobacco mesophyll protoplasts. The *cis*-acting regulatory elements related to stress or hormone responses were predicted in the promoter region of *HbGR2*. The results from real-time RT-PCR analyses showed that *HbGR2* was expressed throughout different tissues and developmental stages of leaves. Besides being related to tapping panel dryness (TPD), *HbGR2* was regulated by several treatments including ethephon (ET), methyl jasmonate (MeJA), drought, low temperature, high salt, wounding and hydrogen peroxide (H_2O_2). The *Escherichia coli* (*E. coli*) cells overexpressing *HbGR2* markedly increased their tolerance and survival at high concentrations of H_2O_2, suggesting that *HbGR2* might play an important role in oxidative stress response in *Hevea brasiliensis*.

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1. Introduction

Glutathione (L-γ-glutamyl-L-cysteinyl-glycine, GSH), a principal low molecular weight tripeptide, is the most abundant source of non-protein thiols in plant cells. It plays an important role in protecting plants against various types of stress caused by reactive oxygen species (ROS) (Alscher, 1989). GSH is part of the ascorbate-glutathione (AsA-GSH) cycle, which is essential in eliminating hydrogen peroxide (H_2O_2) and regenerating AsA. It can be used directly as a free radical scavenger by reacting with superoxide (O_2^-), singlet oxygen ($^1O_2$) and hydroxyl radicals ($OH^{\cdot}$). It also prevents the denaturation of proteins caused by the oxidation of protein thiol groups under stress. As the substrates for glutathione peroxidase (GPX) and glutathione-S-transferases (GST), GSH is involved in the removal of ROS. By maintaining the reduced state of α-tocopherol and zeaxanthin, GSH participates indirectly in protecting membranes. In addition, GSH is involved in the regulation of gene expression, cell signaling, cell cycle, cell death and senescence, plant development, pathogen resistance and enzymatic regulation (Noctor et al., 2002). Within the cells, GSH exists in two different forms: the reduced form (GSH) and the oxidized form (glutathione disulfide, GSSG). The physiological functions of GSH were mainly attributed to its reduced form in plants (Alscher, 1989).

Glutathione reductase (GR; EC 1.8.1.7) is a flavoprotein oxidoreductase and has been found in animals, bacteria, fungi and plants. It catalyzes the reaction of GSSG to GSH using NAD(P)H as an electron donor (Meister and Anderson, 1983), and thus a highly reduced state of GSH and ASA is maintained during oxidative stress. Since plant GR was firstly reported in 1951 (Conn and Vennesland, 1951), a lot of GRs have been purified and characterized from various plants, including *zea mays* (Mahan and Burke, 1987), *Pisum sativum* (Creissen et al., 1991; Stevens et al., 1997), *Arabidopsis thaliana* (Kubo et al., 1993), *Nicotiana tabacum* (Creissen and

Abbreviations: ABA, abscisic acid; bp, base pairs; cDNA, complementary DNA; CTAB, hexadecyltrimethylammonium bromide; EST, expression sequence tag; FAD, flavin adenine dinucleotide; GA, gibberellin; IPTG, isopropyl-ß-D-thiogalactopyranoside; NADH, reduced nicotinamide adenine dinucleotide; NADP(H), reduced nicotinamide adenine dinucleotide phosphate; OD, optical density; ORF, open reading frame; RACE, rapid amplification of cDNA ends; PCR, polymerase chain reaction; RT-PCR, reverse-transcription PCR; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; UTR, untranslated region.

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http://dx.doi.org/10.1016/j.plaphy.2015.07.022
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Mullineaux, 1995), *Oryza sativa* (Kaminaka et al., 1998; Wu et al., 2013), *Populus trichocarpa* (Rouhier et al., 2006) and *Vigna unguiculata* (Contour-Ansel et al., 2006). Most of GRs were localized in chloroplast (Connell and Mullet, 1986), but some were also found in cytosol, mitochondria and peroxisomes (Edwards et al., 1990; Jiménez et al., 1997; Romero-Puertas et al., 2006). According to the presence of N-terminal extension, the GRs were divided into two classes; GR1 and GR2 were the shorter cytosolic and elongated organellar protein, respectively (Rouhier et al., 2006). GRs are encoded by a small gene family in higher plants. Two genes were identified in *A. thaliana* (Kubo et al., 1993), *N. tabacum* (Creissen and Mullineaux, 1995) and *V. unguiculata* (Contour-Ansel et al., 2006), and three genes were found in the genomes of *O. sativa* and *P. trichocarpa* (Rouhier et al., 2006; Wu et al., 2013). *GR* plays an important role in biotic and abiotic stresses response by maintaining the intracellular pool of GSH and ASC. It was reported that *GR* was regulated by stresses and phytohormones such as wounding (Romero-Puertas et al., 2006), ozone (Kubo et al., 1995), chilling (Hodges et al., 1997; Walker and McKersie, 1993), methyl viologen (Le Martret et al., 2011), drought (Contour-Ansel et al., 2006; Torres-Franklin et al., 2008), heavy metals (Le Martret et al., 2011; Xiang and Oliver, 1989; Yannarelli et al., 2007), salinity (Le Martret et al., 2011; Wu et al., 2013) and jasmonic acid (Xiang and Oliver, 1989).

Hevea brasiliensis, commonly known as rubber tree, is a perennial plant that belongs to the Euphorbiaceae family. It is of major economic importance because its latex is primary source of natural rubber. Over-tapping or excessive ethephon stimulation can cause oxidative stress in the laticifer cells (Chrestin, 1989; Fan and Yang, 1984). Excessive ROS can damage cellular membranes and lutoids that contain coagulant factors involved in the aggregation of rubber particles (Chrestin et al., 1984). Furthermore, the destabilized membrane of lutoids leaded to lutoids burst and consecutive in situ latex coagulation. Tapping panel dryness (TPD) is one of the most serious threats to latex yield in rubber tree. Once TPD occurs, the tapping panel region of rubber tree might be partly or entirely dry (no latex flow). It was reported that an uncompensated oxidative stress might be involved in the onset of TPD in rubber tree (Faridah et al., 1996). In TPD rubber tree, the contents of thiols (reduced glutathion) decreased (Fan and Yang, 1984), and a cytosolic GR transcript was down-regulated (Li et al., 2010). A cytosolic GR gene (*HbGR1*) was cloned in rubber tree, and *HbGR1* transcript was constitutively expressed in the latex, leaves, barks and flowers. In addition, *HbGR1* was regulated by ET, MeJA, H_2O_2 and wounding, suggesting that *HbGR1* may be involved in ethylene, MeJA, and stress response in rubber tree (Deng et al., 2014). In this study, we cloned a new GR gene from *Hevea brasiliensis*, named as *HbGR2*. The gene organization, *cis*-acting regulatory elements, phylogenetic tree and expression profiles of *HbGR2* were analyzed in detail. The antioxidant activity of HbGR2 was examined by *in vivo* H_2O_2 tolerance activity assay.

2. Materials and methods

2.1. Plant materials

Rubber tree clone, Reyan 7-33-97, was cultivated at the experimental farm of Chinese Academy of Tropical Agricultural Sciences in Danzhou city, Hainan province, China. In this study, the latex, leaf, bark, female flower, and male flower samples were collected from 20-year-old rubber tree, which was regularly tapped on the s/2 d/4 system with 1.5% ethephon (ET) stimulation for the past 10 years. The seven-year-old virgin trees were treated with 1% ET, 1% methyl jasmonate (MeJA) and 2% hydrogen peroxide (H_2O_2) prior to the first tapping according to the method of Hao and Wu (2000). At the same time, another batch of rubber trees were treated with 1% carboxyl methyl cellulose, lanolin paste, and 1 mM phosphate buffer solution (pH 6.0) as the controls of ET, MeJA and H_2O_2, respectively. The wounding treatment and control were performed as described by Tang et al. (2010). The first few drops of latex containing the debris were discarded, and then the latex from the treated and control rubber tree was allowed to drop directly into liquid nitrogen in an ice kettle for total RNA extraction. The tissue culture seedlings of Reyan 7-33-97 were separately treated with high salt, low temperature and drought as described by Li et al. (2013). The leaves from treated and control seedlings were collected at different time for RNA extraction, respectively. The different developmental leaves were collected from six-year-old Reyan 7-33-97. The latex and barks from healthy and TPD rubber trees were collected for RNA extraction, and the healthy and TPD rubber trees were selected according to Li et al. (2010).

2.2. Isolation of RNA and DNA

Total RNA was isolated from different tissues according to the methods of Xu et al. (2010) and treated with RQ1 RNase-free DNase (Promega, USA). Genomic DNA was extracted from young leaves with the CTAB method (Porebski et al., 1997) and treated with RNase A (Takara, Japan). The integrities and concentration of RNA and DNA were evaluated by agarose gel electrophoresis and determined by a spectrophotometer.

2.3. cDNA cloning of GR2 from Hevea brasiliensis

Total RNA from the latex was used for the synthesis of first-strand cDNA as described for the SMART™ RACE cDNA amplification kit (Clontech, USA). Based on the EST sequence (GenBank accession No.: JR348361), the gene-specific primers for 3′- and 5′-RACE were designed, respectively (Table 1). All PCR products were cloned in pMD18-T vector (Takara, Japan) and sequenced. After aligning and assembling of the EST sequence, 5′-RACE and 3′-RACE products, the full-length cDNA sequence of the *HbGR2* was obtained and testified by PCR amplification with primers HbGR2-UF and HbGR2-UR (Table 1). The PCR was performed using Pyrobest® DNA polymerase (Takara, Japan) with the following conditions: 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 3 min, with a final extension at 72 °C for 10 min. The PCR product was cloned into the pMD18-T vector and sequenced.

2.4. Isolation of the genomic and promoter sequences of HbGR2

The genomic sequence of *HbGR2* was isolated by PCR amplification using LA Taq DNA polymerase (Takara, Japan) with primers HbGR2-UF and HbGR2-UR (Table 1). The reaction was performed with the following conditions: 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 50 °C for 1 min and 72 °C for 6 min, with a final extension at 72 °C for 10 min. The promoter sequence of *HbGR2* was isolated by PCR amplification of the genomic DNA using GenomeWalker universal kit (Clontech, USA). The primary PCR was performed with the outer gene-specific primer HbGR2-GSP1R (Table 1) and the outer adaptor primer (AP1) using Advantage2 polymerase mix (Clontech, USA). The secondary PCR was performed with the nested gene-specific primer HbGR2-GSP2R (Table 1) and the nested adaptor primer (AP2). The PCR product was cloned into the pMD18-T vector and sequenced.

2.5. Multiple sequence alignment and bioinformatics analyses

The conserved domains and subcellular localization of HbGR2 were separately predicted with SMART (http://smart.embl-

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