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Research article

The function of *Rad6* gene in *Hevea brasiliensis* extends beyond DNA repair

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ABSTRACT

The *Rad6* gene of *Saccharomyces cerevisiae* encodes an ubiquitin-conjugating enzyme (E2) which is required for DNA repair, damage-induced mutagenesis, sporulation, etc. In this study, one *Rad6* homolog, designated *HbRad6*, was cloned in rubber tree (*Hevea brasiliensis*). The putative protein sequence of *HbRad6* contains 152 amino acids, a conserved UBC domain, and a conserved active-site cysteine in the UBC domain, which is required for E2 enzymes catalytic activity. HbRad6 shared high similarity with Rad6 from other species. It shared the highest similarity with rice OsRad6 and *Arabidopsis thaliana* AtUBC2 with 96.05% identical residues, and 63.16% sequence identity with yeast Rad6 (excluding the acidic tail). Comparing expression among different *Hevea* tissues demonstrated that *HbRad6* was ubiquitously expressed in all tissues, but it revealed a preferential expression in the latex. Furthermore, *HbRad6* expression was markedly induced by DNA-damaging agent H_2O_2, the latex stimulator ethephon (ET), and methyl jasmonate (MeJA), while NaCl and wounding treatments had relatively minor effect upon its expression. Genetic complementation experiment revealed that *HbRad6* had minor effects on the complementation of the UV sensitivity of yeast *rad6* null mutant, indicating that the *Hevea* Rad6 protein may partially suppress the UV sensitivity of the yeast *rad6* mutant. These results suggested that *HbRad6* was a multifunction gene involved in DNA damage repair, hormones and stress responses in rubber tree.

1. Introduction

Protein ubiquitination is a posttranslational modification that regulates many important cellular processes [1]. Ubiquitination is a multistep reaction, sequentially involving three enzymes referred to as an E1 (ubiquitin-activating enzyme, UBA), an E2 (ubiquitin-conjugating enzyme, UBC), and an E3 (ubiquitin ligase) [2]. The first event in the cascade is the ATP-dependent formation of a thioester-linked ubiquitin by E1. The thioester-linked ubiquitin is then transferred to the cysteine residue of E2s. E3s facilitate the transfer of ubiquitin to a lysyl group on the substrate. E3s mediate this step either through the formation of an ubiquitin thioester prior to transfer to the substrate or by noncovalent interaction with E2s carrying the thioester-linked ubiquitin. The E2 family is characterized by the presence of a highly conserved ubiquitin-conjugating (UBC) domain, and a conserved cysteine residue in the UBC domain. E2s act via selective protein–protein interactions with the E1 and E3s and connect activation to covalent modification. By doing so, E2s differentiate effects on downstream substrates, either with a single Ub/Ub-like molecule or as a chain [3].

Among E2 family, the most interesting class is the one homologous to Rad6 (UBC2) from yeast (*Saccharomyces cerevisiae*). The *Rad6* gene in *S. cerevisiae* encodes a 172-amino acid and 20-kDa E2 enzyme, with a UBC domain consisting of approximately the first 149 amino acids and a extended 23-residue, predominantly acidic C terminus [4]. Rad6 ubiquitin-conjugating enzyme acts together with E3 Ubr1 in N-end rule degradation [5], with E3 Rad18 in monoubiquitination of the DNA replication factor PCNA [6], and with E3 Bre1 in monoubiquitination of histone H2B [7]. The yeast *Rad6* is essential for a variety of chromosomal processes. Yeast *rad6* mutants display a pleiotropic phenotype including defects in DNA repair, proteolysis of N-end rule protein substrates, cellular proliferation, cell cycle progression, and an inability to sporulate [8]. The *Rad6* family is highly conserved from yeast to mammals. According to the sequence similarity, one or several homologs of *Rad6* have been identified in other eukaryotes. In human, two *Rad6* homologs have been identified, designated *HHR6A* and *HHR6B*, and both of them can carry out the DNA repair and mutagenesis functions of *Rad6* in *S. cerevisiae rad6* mutants [9]. Mouse *HR6A* is highly homologous to *Rad6*. The *mHR6A*-deficient cells have normal DNA damage resistance properties, but *mHR6A* knockout male and

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female mice display a small decrease in body weight [10]. Three *Rad6* homologs, *AtUBC1*, *AtUBC2*, and *AtUBC3*, exist in *Arabidopsis* genome [11]. Functional tests revealed that *AtUBC2* can partially rescue the UV sensitivity and reduce growth rate of *rad6* mutants at elevated temperatures [12]. *AtUBC1* and *AtUBC2* redundantly mediated histone H2B monoubiquitination, and upregulated the expression of *FLOWERING LOCUS C* (*FLC*; a central flowering repressor in *Arabidopsis*) and *FLC* relatives, and also redundantly repressed flowering, but *AtUBC3* was not involved in regulating flowering time [11,13]. Soybean *GmUBC2* was up-regulated by drought and salt stress. Overexpression of *GmUBC2* conferred enhanced drought and salt tolerance through modulating abiotic stress-responsive gene expression in *Arabidopsis* [14]. Rad6 from *Oryza sativa* L. cv. Nipponbare (OsRad6) interacted with OsSgt1 which is a subunit of the SCF ubiquitin ligase complex. Both of *OsRad6* and *OsSgt1* were strongly expressed only in the proliferating tissues and induced by UV- and DNA-damaging agents such as MMS or H_2O_2. OsRad6 was related not only to post-replicational repair but also to the proteasome system [15].

Although *Rad6* homologs have been identified in several annual plant species, little is known about their roles in tree species. Rubber tree (*Hevea brasiliensis*) is a tropical perennial tree for the production of natural rubber. Rubber molecules are produced, aggregated and packaged in the latex vessels (laticifers) of rubber tree. The latex, a cytoplasmic component of the laticifers, expels from the laticifers upon tapping. TPD (tapping panel dryness) is the appearance of partial or complete stoppage of latex flow along the tapping panel. The latex yield in plantations faces serious economic losses caused by TPD. In previous study, an ubiquitin-conjugating enzyme gene (GeneBank accession number: GO349182) which homologs to *Rad6* was identified and significantly up-regulated in TPD trees using suppression subtractive hybridization (SSH) method [16]. In order to characterize the biological function of *Rad6* gene in rubber tree, the *Hevea Rad6* gene was cloned, and its DNA repair function and expression profiles were systematically analyzed in this study.

2. Results

2.1. Isolation of Hevea Rad6 and analyses of its gene structure and putative protein domain architecture

In previous study, the partial sequence of E2 with high similarity to *Rad6* (GeneBank accession number: GO349182) was identified to significantly up-regulate in TPD rubber tree [16]. The sequence of GO349182 was firstly used to search the NCBI nr nucleotide database and *de novo* assembly of transcriptome sequencing data [17], then the homologous sequences were assembled and used to design primers for RACE amplifications. Two distinct sequences were obtained by RACE methods. Both of them encoded homologous protein Rad6, and one sequence named as *HbRad6* was chosen for further study. The full-length cDNA of *HbRad6* was 929 bp in length (excluding 27 bp of poly(A) tail). It contained a 459-bp ORF encoding a putative protein of 152 amino acids, flanked by a 245-bp 5′-UTR and a 225-bp 3′-UTR. The predicted molecular mass of HbRad6 was 17.31 kDa, with a pI of 5.37. Based on structural properties indicated by the NCBI Conserved Domain search (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi?) and SMART (http://smart.embl-heidelberg.de/), the predicted protein was a ubiquitin-conjugating enzyme E2 and a homolog of yeast Rad6. The predicted protein HbRad6 had a conserved UBC domain (from the 7th to 150th amino acid), with a conserved active-site cysteine in the UBC domain, which is required for E2 enzymes catalytic activity. However, HbRad6 hasn't the acidic C-terminal sequence present in the yeast Rad6 protein, which is required for the sporulation function but not for the DNA repair and induced mutagenesis functions of Rad6 [18].

To reveal the gene structure of *HbRad6*, primers spanning the full-length cDNA were used to amplify the genomic DNA, and confirmed by sequenced the amplified product. Comparing the genomic sequence with the corresponding cDNA sequence, it showed that the genomic sequence of *HbRad6* was 4181 bp in length and contained five introns, with one in 5′-UTR and the other four in ORF region (Fig. 1). The gene structure of *HbRad6* is similar to the *Arabidopsis thaliana Rad6* homolog *AtUBC2*, which also has an intron in the 5′-UTR sequence and four present in the ORF [12].

2.2. Multiple sequence alignment and phylogenetic analysis of the Rad6 family from various species

To determine the evolutionary relationship of *HbRad6* with *Rad6s* from other species, the phylogenetic tree was constructed by the neighbor-joining method. As shown in Fig. 2a, the Rad6 family was highly conserved among all the kingdoms. A conserved active-site cysteine required for E2 enzymes catalytic activity was well retained in all the Rad6 members (Fig. 2a, indicated by the asterisk). HbRad6 shared the highest similarity with rice OsRad6 and *A. thaliana* AtUBC2 with 96.05% identical residues, 94.74% identity with *A. thaliana* AtUBC1, over 73% identity with *Homo sapiens* HHR6A and HHR6B, and 63.16% identity with the *S. cerevisiae* Rad6 (excluding the acidic tail). AtUBC3 diverged from the branch containing AtUBC1, AtUBC2 and proteins from other plant species, and shared 86.84% identity with HbRad6 (Fig. 2b).

2.3. Expression analysis of HbRad6 in rubber tree

The expression profiles of *HbRad6* were systematically analyzed in rubber tree. Comparing expression among different *Hevea* tissues demonstrated that *HbRad6* was ubiquitously expressed in all tissues, but of the six tissues tested, it revealed a preferential expression in the latex (7.5-fold over in the bark tissue), suggesting its major role in this tissue (Fig. 3a). Since some *Rad6* homologs were regulated by the DNA-damaging agents and stresses, the *HbRad6* transcript was detected under H_2O_2, NaCl and wounding treatments. As expected, the *HbRad6* transcript was significantly induced by H_2O_2 treatment, reaching the maximum level at 48 h (4.3-fold over the untreated control (0 h)) (Fig. 3b). Salt stress (treated with 1 mol L^{-1} NaCl) and wounding treatments had relatively minor effect upon the *HbRad6* expression, and the maximum levels occurred at 3 h and 24 h, respectively (Fig. 3c and d). The preferential expression of *HbRad6* in the latex indicated that *HbRad6* might be involved in regulating latex regeneration and/or flow. Meanwhile, ET and JA are associated with latex yield in rubber tree. So the expression patterns of *HbRad6* under ET and MeJA

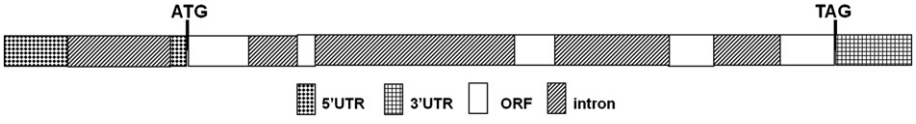

Fig. 1. Schematic diagram of the gene structure of *HbRad6*.