

Identification of a Highly Expressed 3-Hydroxy-3-Methylglutaryl-CoA Reductase Gene in the Root Tissue of *Taraxacum kok-saghyz*

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Abstract

Kazakh dandelion (*Taraxacum kok-saghyz*, *Tk*) is a rubber-producing plant currently being investigated as a source of natural rubber for industrial applications. Like many other isoprenoids, rubber is a downstream product of the mevalonate pathway. The 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) enzyme catalyzes the conversion of 3-hydroxy-3-methylglutaryl-CoA to mevalonic acid, a key regulatory step in the MVA pathway. Such regulated steps provide targets for increases in isoprenoid and rubber contents via genetic engineering to increase enzyme activities. In this study, we identify a *TkHMGR1* gene that is highly expressed in the roots of Kazakh dandelion, the main tissue where rubber is synthesized and stored. This finding paves the way for further molecular and genetic studies of the *TkHMGR1* gene, and its role in rubber biosynthesis in *Tk* and other rubber-producing plants.

Keywords

3-Hydroxy-3-Methylglutaryl-CoA Reductase, *Taraxacum kok-saghyz*, Root, Gene Expression, Quantitative Polymerase Chain Reaction

1. Introduction

The main source of natural rubber in the world is the rubber tree *Hevea brasiliensis* grown mainly in Southeast Asia [1] [2]. Other rubber-producing plants, such as Kazakh dandelion (formerly Russian dandelion)

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(*Taraxacum kok-saghyz*, *Tk*) and Guayule (*Parthenium argentatum*) are currently being developed as alternative crop sources of natural rubber [3]-[6].

Biosynthesis of rubber starts with an allylic pyrophosphate initiator, such as farnesyl pyrophosphate (FPP), followed by progressive addition of isopentenyl pyrophosphate (IPP) molecules [7]-[9]. The initiator and IPP molecules are synthesized by the mevalonate (MVA) pathway in the cytosol of plant cells [10] [11]. IPP is also synthesized in plastids via the 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway [8] [11] [12]. The 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) is a key regulatory enzyme in the MVA pathway [13]-[16]. In all plants studied so far, HMGR is encoded by multiple genes, ranging from two in Arabidopsis to eight in cotton [17]. The different plant isoforms are commonly regulated at the transcriptional level [11] [18] [19]. Only a few cases of translational [20] and post-translational [21] [22] regulation have been reported. Among rubber producing plants, three HMGR isoforms have been cloned from Hevea, with isoform *HbHMGR1* (NCBI Genbank accession number: X54659) associated with rubber biosynthesis [23] [24]. In *Taraxacum brevicorniculatum*, three HMGR isoforms have been characterized, each with distinct tissue expression profiles. Among them, *TbHMGR1* is the only isoform expressed in latex (the milky cytoplasm of the highly differentiated cells called laticifers) where rubber is synthesized [25]. Recently, one *TkHMGR* (HQ857601) sequence was released in GenBank, but it was not characterized.

Constitutive over expression of *HMGR* genes has proven to be effective in increasing production of selected isoprenoids and end-product sterols in plants [26]-[28]. The main organ of rubber synthesis and storage in *Tk* is the root. To increase rubber biosynthesis in *Tk* by means of genetic engineering, one approach is to over express a *HMGR* in the root tissue. Here we report the identification of a root-specific *TkHMGR1* through data mining and gene expression studies.

2. Materials and Methods

2.1. Plant Material

Tk seeds were obtained from the United States Department of Agriculture-Agricultural Research Service National Plant Germplasm System, where they were deposited after collection in Kazakhstan in 2008 (accession No. KAZ08-014, ID W6-35169). Plants were germinated and grown in a greenhouse at temperatures between 28°C (day) and 18°C (night), with supplemental metal halide lighting to provide a 15-h-day length (1000 to 1250 $\mu\text{mol m}^{-2}\cdot\text{s}^{-1}$).

2.2. RNA Extraction and Quantitative PCR Analysis

Total RNA from root, leaf and flower tissues of greenhouse-grown *Tk* plants was extracted using a PowerPlant[®] RNA Isolation Kit with DNase (MoBio Laboratories Inc., USA). The total RNA was converted to cDNA using the Superscript III First-Strand Synthesis System for Reverse Transcriptase (RT)-Polymerase Chain Reaction (PCR) (Life Technologies, USA). Quantitative PCR (qPCR) on cDNAs of *TkHMGR* and 18S *rRNA* was performed using Applied Biosystems 7500 Fast Real Time PCR System. The following combinations of forward/reverse primers were used for the qPCR reactions:

for *TkHMGR1* 5'-CTGGA ACTATTGCTCCGACAA-3'/5'-GCCATGGCGGACCCTGTCAGG-3' and for *Tk18S rRNA* 5'-CGGCTACCACATCCAAGGAA-3'/5'-TGTC ACTACCTCCCCGTGTCA-3'.

Other primer sets used in spatial and temporal analysis of *Tk HMGR*-like sequences are:

TkHMGR2 5'-TTTGA AATCTTCTTGGAGGAA-3'/5'-ACCATGTTTCATCCCCATTGCATC-3';
TkHMGR3 5'-TTGCCACGGGACAAGACCCCGCT-3'/5'-GACTGTGAAGCTAGTTGAGTTCC-3';
TkHMGR4 5'-CGGTCAGACCCTGCCCGGAATGTG-3'/5'-TCTATGGAAGGCATGGTGACAGA-3';
TkHMGR5 5'-TTGCAGCACCGGTGATGCAATGGG-3'/5'-ACAGACTTCCACGCCCTTCTATCCA-3'.

PCR amplification reactions were carried out in a volume of 25 μL containing 20 ng of cDNA, 0.5 μM each of forward and reverse primers, and 1x SYBR[®] Green Master Mix (Applied Biosystems USA), using the following temperature regime: 95°C for 20 s, followed by 40 cycles of 95°C for 3 s and 60°C for 30 s. The relative copy numbers of a gene were averaged over triplicates in the same 96-well micro chamber plate. Expression of *TkHMGR* was normalized to the expression of the constitutively expressed endogenous reference gene 18S *rRNA*. The method of Pfaffl (2001) [29] was applied to calculate comparative expression levels between samples. The qPCR experiments were repeated at least twice.

3. Results and Discussion

3.1. Computational Analysis of *TkHMGR*-Like Expressed Sequences

Hevea *HMGR1* (X54659) was used as query for homology searches (tblastn) of a *Tk* root tissue expression library DNA sequences available at the National Center for Biotechnology Information (NCBI). Seventeen *TkHMGR*-like partial sequences were found with an average length of 750 bp (**Table 1**). Eleven of the sequences were homologous to the N-terminus of Hevea *HMGR1*, three to the C-terminus, and three to the middle portion of the protein (between amino acids 167 - 426). The large numbers of N-terminus homology sequences and the low representation of sequences with C-terminus homology suggest a technical problem during construction of the cDNA library. *HMGR* is known to be present in plants in several isoforms. Without full-length *TkHMGR* sequence information, it is impossible to determine the number of *TkHMGR* isoforms. However, spatial (organ specific) and temporal (age specific) expression analysis of these sequences can provide useful information about the presence of tissue-specific isoforms.

3.2. Spatial and Temporal Analysis of *TkHMGR*-Like Sequences

We aligned all 17 *Tk* sequences against the Hevea *HMGR1* gene (X54659) and designed five sets of PCR primers. Computational analysis of the five primer sets predicted that each would amplify at least one of the following 6 sequences, GO661008, GO662289, GO668051, GO666861, GO665221, and GO670200. PCR amplifications were performed on cDNAs derived from total RNA extracted from leaf, root, and flower tissues of ~11 weeks old *Tk* plants. All primers sets amplified a product in all tissues (data not shown). However one primer set (Materials and Methods) consistently amplified an abundant product from cDNAs from root tissues. This primer set, herein called *TkHMGR1* primers, was used for quantitative expression analysis. Computational analysis of *TkHMGR1* primers predicted only one PCR product from only one sequence, GO666861, and, interestingly, no amplification from the cloned *TkHMGR* sequence (HQ857601).

Table 1. List of *TkHMGR*-like sequences showing homology to Hevea *HMGR1*.

Accession No.	% Coverage*	Homology location**	% Identity	Length (bases)
GO661008	42	Center	85	768
GO662289	40	Center	83	760
GO668051	44	Center	85	773
GO661429	45	N-term	67	783
GO666861	33	C-term	89	655
GO661610	36	N-term	60	786
GO660958	38	N-term	57	750
GO661845	37	N-term	58	807
GO661812	35	N-term	59	775
GO662014	34	N-term	58	752
GO665221	21	C-term	85	671
GO661926	36	N-term	52	762
GO670200	19	C-term	88	665
GO660861	34	N-term	53	754
GO662370	34	N-term	53	781
GO661179	33	N-term	53	758
GO662172	32	N-term	52	725

*Compared to Hevea *HMGR1* sequence (X54659); **Location in Hevea *HMGR1* sequence (X54659).

To examine the relationship between rubber accumulation and *TkHMGR1* expression, we further quantified *TkHMGR1* transcript levels in root and leaf of young (~11 weeks old) and mature (~20 weeks old) *Tk* plants using qPCR technology. In our lab, rubber extractions from *Tk* roots typically yield 4 mg/g fresh weight from 12-week-old plants and 10 mg/g fresh weight from 20-week old plants). No rubber was detected in leaf tissues. As shown in **Figure 1**, we detected 11-fold or 3-fold higher of *TkHMGR1* transcript levels in roots than in leaves from young or mature plants, respectively. The results indicated that the expression of *TkHMGR1* was higher in root than leaf during the active growth phase of *Tk* plants. Although functional studies need to be carried out in order to determine the enzymatic activity and substrate specificity of *TkHMGR1* in rubber metabolism, it is likely that *TkHMGR1* contributes to rubber synthesis in *Tk* roots based on its expression profile.

Rubber is the end metabolite of the MVA biosynthesis pathway. The amount of rubber in mature root is the result of continuous synthesis and accumulation over the lifespan of the plant. Although we observed a decline in expression of *TkHMGR1* in roots when plants became mature (**Figure 1**), the level of the transcript was probably still high enough to produce active *TkHMGR1* enzymes for rubber biosynthesis. It is also possible that *TkHMGR1* is a relatively stable protein, and thus supports continuous biosynthesis of rubber over development, leading to the high accumulation of rubber in root.

In summary, we have identified the *TkHMGR1* isoform that is highly expressed in *Tk* root. The C-terminal part of *TkHMGR1* sequences are represented by GenBank accession GO666861. The partial sequence provided the basis for the design of isoform-specific primers that can be used to isolate full-length *TkHMGR1* and eventually its promoter sequences. The likely role that *TkHMGR1* plays in rubber synthesis suggests that its over-expression in guayule or *Tk* could increase rubber production. In addition, the *TkHMGR* promoter can be used to drive gene expression for other key enzymes/proteins associated with rubber synthesis in *Tk* root, such as the genes for IPP synthase, cis-prenyltransferase, rubber elongation factor, etc. Thus, the sequence identified in this study could lead to other valuable tools for genetic engineering of rubber-producing crops.

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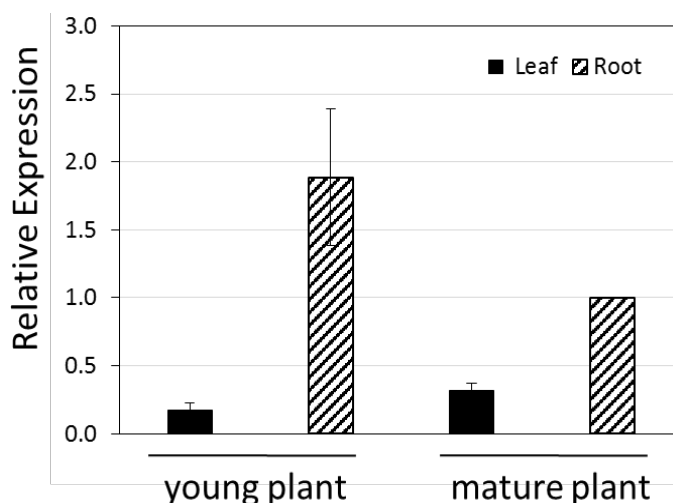


Figure 1. Expression of *TkHMGR1* in root and leaf of young (11 week) and mature (20 week) *Tk* greenhouse plants. Expression levels determined by qPCR are relative to those of 18S ribosomal RNA in the same samples. Each data point represents the mean \pm SD of three replicates.

Department of Agriculture does not constitute an endorsement and does not imply a recommendation over other suitable products.

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