Phytochemistry 79 (2012) 46–56

Contents lists available at SciVerse ScienceDirect

Phytochemistry

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

Altered levels of the *Taraxacum kok-saghyz* (Russian dandelion) small rubber particle protein, TkSRPP3, result in qualitative and quantitative changes in rubber metabolism

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ARTICLE INFO

Article history:
Received 27 September 2011
Received in revised form 15 March 2012
Available online 19 May 2012

Keywords:
Russian dandelion
Taraxacum kok-saghyz
Rubber
Asteraceae
Small rubber particle protein
Rubber transferase

ABSTRACT

Several proteins have been identified and implicated in natural rubber biosynthesis, one of which, the small rubber particle protein (SRPP), was originally identified in *Hevea brasiliensis* as an abundant protein associated with cytosolic vesicles known as rubber particles. While previous *in vitro* studies suggest that SRPP plays a role in rubber biosynthesis, *in vivo* evidence is lacking to support this hypothesis. To address this issue, a transgene approach was taken in *Taraxacum kok-saghyz* (Russian dandelion or Tk) to determine if altered SRPP levels would influence rubber biosynthesis. Three dandelion SRPPs were found to be highly abundant on dandelion rubber particles. The most abundant particle associated SRPP, TkSRPP3, showed temporal and spatial patterns of expression consistent with patterns of natural rubber accumulation in dandelion. To confirm its role in rubber biosynthesis, *TkSRPP3* expression was altered in Russian dandelion using over-expression and RNAi methods. While *TkSRPP3* over-expressing lines had slightly higher levels of rubber in their roots, relative to the control, *TkSRPP3* RNAi lines showed significant decreases in root rubber content and produced dramatically lower molecular weight rubber than the control line. Not only do results here provide *in vivo* evidence of TkSRPP proteins affecting the amount of rubber in dandelion root, but they also suggest a function in regulating the molecular weight of the *cis*-1, 4-polyisoprene polymer.

1. Introduction

Natural rubber (*cis*-1, 4-polyisoprene) is a vitally important and renewable raw material used in the manufacturing of industrial and medical products. High quality natural rubber is a uniform high molecular weight polymer (i.e., >1000 kilodaltons (kDa) or 1000 kilograms per mole (kg/mol)) that cannot be replaced by synthetics in most applications. Of the over 2500 plants that produce natural rubber, only a small subset produce significant quantities of high molecular weight rubber to be economically viable (Mooibroek and Cornish, 2000). Currently, the main commercial source of natural rubber is *Hevea brasiliensis* (*Hevea*). However, *Parthenium argentatum* (guayule) and *Taraxacum kok-saghyz* (Russian dandelion or Tk), which both also produce copious amounts of high quality rubber, are being developed as alternative sources of commercial grade natural rubber (Mooibroek and Cornish, 2000; van Beilen and Poirier, 2007).

In all rubber-producing plant species, a rubber transferase catalyzes the synthesis of a rubber molecule from a single allylic pyrophosphate (APP) primer molecule to which isopentenyl-pyrophosphate (IPP) units are progressively added to form a polymer of varying length (Archer and Audley, 1987; Tanaka, 1989). Although several APPs are effective initiators of rubber biosynthesis *in vitro*, (e.g., dimethylallyl-PP (5-carbons), geranyl-PP (*trans*, 10 carbons), and farnesyl-PP (FPP, all-*trans*, 15 carbons)), FPP is believed to be the primary initiator *in vivo* (Cornish and Siler, 1995; Tanaka et al., 1996; Tangpakdee et al., 1997; Xie et al., 2008). While it is known

Abbreviations: Tk, *Taraxacum kok-saghyz*; SRPP, small rubber particle protein; *TkSRPP1-5*, *Taraxacum kok-saghyz* SRPP genes; TkSRPP1-5, *Taraxacum kok-saghyz* SRPP proteins.

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http://dx.doi.org/10.1016/j.phytochem.2012.04.015

that much of the IPP utilized for rubber biosynthesis is derived from the mevalonate pathway, recent gene expression evidence from *Hevea* latex suggests that the plastidic localized 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway may also contribute IPP to rubber biosynthesis (Ko et al., 2003; Chow et al., 2007). After initiation and elongation a termination event occurs, in which the rubber molecule is released from the rubber transferase.

The elongating natural rubber polymer is produced in cytosolic vesicles known as rubber particles, which consist of a monolayer lipid membrane with species-specific proteins associated on the surface, and a hydrophobic rubber core (Backhaus and Walsh, 1983; Cornish et al., 1999a; Cornish, 2001; Wood and Cornish, 2000). NMR spectroscopic analyses established that, in addition to functioning in rubber sequestration, rubber particles are also the site of *de novo* rubber biosynthesis (Tanaka, 1989). When provided with the appropriate substrates, rubber particles are sufficient to incorporate IPP into *cis*-polyisoprene, thus indicating that these vesicles contain all of the necessary enzymatic machinery required for rubber transferase activity (Backhaus and Walsh, 1983; Cornish and Backhaus, 1990; Madhavan et al., 1989; Tanaka et al., 1996).

Unfortunately, due to the fact that the rubber transferase is membrane associated, classic biochemical approaches have failed in the complete purification and reconstitution of this enzymatic complex. This limitation has made it difficult to directly link candidate proteins to the rubber biosynthetic machinery. Even with over half a century of study, the protein subunits of the rubber transferase remain uncertain. However, several candidate proteins have been identified through criteria related to their association with either the rubber particle (Dennis and Light, 1989; Light and Dennis, 1989) or through spatial and temporal patterns of gene expression consistent with natural rubber deposition (Kush et al., 1990; Han et al., 2000; Chow et al., 2007).

One such protein, the small rubber particle protein (SRPP), was first discovered in *Hevea brasiliensis*, where it exists as a low molecular weight (~23 kDa) acidic protein (pI = 4.8) (Oh et al., 1999). Since its discovery, a SRPP homologue has also been identified in guayule (Kim et al., 2004). Several lines of evidence support a role for SRPP in rubber biosynthesis. First, the *Hevea SRPP* gene is highly expressed in the rubber producing latex fraction of *Hevea brasiliensis*, and recent evidence in Russian dandelion also shows high *SRPP* expression in latex (Oh et al., 1999; Schmidt et al., 2009). Second, the SRPP protein is localized and highly abundant on the surface of rubber particles (Yeang et al., 1998). Third, *in vitro* studies have shown that the addition of either recombinant *Hevea* or guayule SRPPs to isolated *Hevea* rubber particles resulted in significant increases in rubber transferase activity (Oh et al., 1999; Kim et al., 2004). And finally, pre-incubating isolated *Hevea* rubber particles with antibodies specific to SRPP resulted in decreased rubber transferase activity relative to untreated controls (Oh et al., 1999). Interestingly, recent studies from Wititsuwannakul et al. (2008) have also identified the *Hevea* SRPP as an important protein factor involved in latex coagulation. It is thought that latex coagulation is a process by which wound sites are sealed to prevent infection by fungal or bacterial pathogens (El Moussaoui et al., 2001). These results indicate a potentially broader role for SRPP in rubber-producing plant species.

While evidence suggests a role for SRPP in rubber transferase activity, *in vivo* support is lacking. Therefore, a reverse genetic approach using transgenic plants was taken to test the hypothesis that SRPP expression levels directly correlate with the quality and quantity of natural rubber produced in plants. For these studies, Russian dandelion, which is readily transformed using *Agrobacterium tumefaciens* (Wahler et al., 2009), was used as the model transgenic system to functionally test candidate genes for their role in rubber biosynthesis. The primary advantage of using dandelion over *Hevea* and guayule, which are both also amenable to *Agrobacterium* mediated transformation (Blanc et al., 2006; Pan et al., 1996; Dong et al., 2006), is that dandelion transformants can be analyzed for altered rubber phenotypes within nine months. *Hevea* and guayule transformants, on the other hand, require five and two years, respectively, before they are mature enough for appreciable levels of rubber production. Additionally, because dandelion is a small herbaceous plant and not a tree or shrub like *Hevea* and guayule, large numbers of plants can be generated and propagated within environmentally-controlled growth systems. These features make Russian dandelion the most rapid and practical means of studying plant rubber metabolism through a transgenic approach.

In this study, members of the dandelion *SRPP* gene family were further characterized in relationship to rubber biosynthesis. Specifically, *TkSRPP3* gene expression was correlated with spatial and tissue specific patterns of natural rubber accumulation. Furthermore, by altering *TkSRPP3* gene expression in transgenic dandelion, evidence of an *in vivo* function for TkSRPPs is shown.

2. Results

2.1. Identification of rubber particle associated SRPPs

Homology-based searches of a Russian dandelion EST collection using the *Hevea* SRPP as query led to the identification of five cDNAs encoding putative Russian dandelion *SRPP* genes (Schmidt et al., 2009). Proteomic analyses established that three of these SRPP isoforms, specifically TkSRPP3, TkSRPP4, and TkSRPP5, were associated with Russian dandelion rubber particles (Fig. 1, Table 1). These three SRPPs migrate as a tight cluster when analyzed by 2D SDS PAGE (Fig. 1). The identity of each SRPP spot was assigned with a high degree of confidence due to the high quality spectra generated from mass spectrometry analyses. As such, over 80% sequence coverage was obtained for TkSRPP3 and over 40% sequence coverage for TkSRPP4 and 5 (Table 1). Based on Sypro® Ruby protein staining intensity of 2D gels, TkSRPP3 appeared to be the most abundant of the three rubber particle associated SRPPs with TkSRPP5 and TkSRPP4 being present at progressively lower levels. Because TkSRPP3 appeared to be the predominant rubber particle isoform, it was chosen as the focus of this research.

2.2. Assessing TkSRPP mRNA and protein levels

To assess *TkSRPP* transcript levels, a quantitative real time RT-PCR approach was taken. Unfortunately, while it is often possible to assess mRNA levels of individual gene family members using real time-PCR, this proved difficult in our case due to the high degree of nucleotide sequence identity shared between the three particle associated *TkSRPPs*. Therefore, while it was possible to develop a TaqMan quantitative real time RT-PCR assay that specifically measured *TkSRPP3* levels, it was not possible to develop assays to specifically determine *TkSRPP4* and *TkSRPP5* mRNA levels. To evaluate TkSRPP protein levels, an affinity purified polyclonal antibody was generated using recombinant TkSRPP3. Western blot analysis of 2D-PAGE fractionated rubber particle proteins indicated that this antibody showed high avidity towards TkSRPP3 and 5, and fairly low avidity towards TkSRPP4 (Supplementary Fig. S1). Therefore, while the antibody was useful for determining levels of two of the major rubber particle isoforms, it could not be used to reliably detect TkSRPP4.

2.3. TkSRPP3 expression correlates spatially and temporally to dandelion root rubber production

TkSRPP3 transcript levels in roots were almost 2-fold higher than those observed in leaf tissue, and over 10-fold higher relative to levels in stem and flowers (Fig. 2A). These results are in agreement with those of Schmidt et al. (2009), where *TkSRPP3* gene