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Identification and comparison of natural rubber from two *Lactuca* species

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Abstract

Renewed interest in the identification of alternative sources of natural rubber to *Hevea brasiliensis* has focused on the Compositae family. In our search for Compositae models for rubber synthesis, we extracted latex from stems of two lettuce species: *Lactuca serriola*, prickly lettuce, and *Lactuca sativa* cv. Salinas, crisphead lettuce. Both species contained *cis*-1,4-polyisoprene rubber in the dichloromethane-soluble portions of their latex, and sesquiterpene lactones in their acetone-soluble portions. The rubber from both species and their progeny had molecular weights in excess of 1,000,000 g/mol, and polydispersity values of 1.1. Rubber transferase activity was detected across a range of farnesyl diphosphate initiator concentrations, with decreased activity as initiator concentrations exceeded putative saturation. These results add lettuce to the short list of plant species that produce high molecular weight rubber in their latex. Due to the genomic and agronomic resources available in lettuce species, they provide the opportunity for further dissection of natural rubber biosynthesis in plants.

Keywords: *Lactuca sativa*; *Lactuca serriola*; Compositae; Rubber; *cis*-1,4-Polyisoprene; *Lactuca*; Lettuce

1. Introduction

Natural rubber refers to a coagulated or precipitated *cis*-1,4-polyisoprene product obtained from the latex of rubber producing plants. Rubber molecules are synthesized in the cytoplasm of plant laticifer cells by a rubber transferase (EC 2.5.1.20), and surrounded by a species-specific fatty-acid monolayer and rubber particle-associated proteins (Siler et al., 1997). The isoprenoid precursors of rubber are considered to be synthesized in the cytosol as part of the general mevalonic acid (MVA) pathway (Kush, 1994; Laule et al., 2003). The *cis*-prenyltransferase activity of a rubber molecule progressively adds isopentenyl-diphosphate (IPP) moieties onto a single allylic diphosphate primer molecule to form a biopolymer. The lengths of the resulting biopolymers determine the functionally important molecular weight of rubber (Bouton, 1992).

Rubber is a vital and strategic raw material utilized in the manufacturing of more than 40,000 consumer products (Cornish, 2001a). Synthetic rubber (predominantly styrene-butadiene polymer) lacks many desirable properties and cannot completely substitute for natural rubber in numerous high performance applications that require properties

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such as high tensile strength and abrasion resistance. The International Rubber Study Group estimated that worldwide natural rubber consumption in 2004 was 8.4 million tons and has consistently outpaced production (http://www.rubberstudy.com). Currently, by far the largest commercial source of natural rubber is *Hevea brasiliensis* (Willd. ex Adr. Juss.) Muell. Arg. (Mooibroek and Cornish, 2000). However, latex allergies triggered by *H. brasiliensis* proteins affect >7% of the population and can be very severe (Rolland et al., 2005). In addition, the *H. brasilinesis* crop consists predominately of plantation-grown clonal trees that lack genetic diversity, thereby rendering the crop vulnerable to pathogenic attack (Le Guen et al., 2004). For these reasons, as well as political and economic instability in some countries producing and exporting *H. brasiliensis* rubber, there is considerable interest in finding alternative commercial sources of natural rubber in crop plants that grow well in northern climates.

Twenty botanical families, 900 genera, and 12,500 species produce latex, of which eight families, 300 genera, or 1800 species, produce rubber in that latex (Metcalfe, 1966). The history of rubber production is replete with attempts to identify alternate sources of rubber, both temperate and tropical (Bowers, 1990; Mitchell et al., 1942; Carr and Bagby, 1987; Buchanan et al., 1978; Anonymous, 1956; Hall and Goodspeed, 1919), but few plant species known to produce rubber are capable of producing large amounts of high molecular weight rubber (Mooibroek and Cornish, 2000). Of those, only *Parthenium argentatum* Gray is currently commercially produced on a small scale (Cornish et al., 2001).

In 1913, Charles Fox reported the analysis of latex from two lettuce species, *Lactuca canadensis* (L.) and *L. scariola* (L.) (Fox, 1913). Both species were found to secrete an abundance of latex from all areas of the plant, especially the stem. Rubber content in the latex was determined to be 2.2% in *L. canadensis* and 1.6% in *L. scariola*. The variability of rubber production among lettuce genotypes was also described by the Clemson Agriculture Centre (Mitchell et al., 1942), and the US Department of Agriculture (Anonymous, 1956). These reports cited differences in rubber content in the latex, of several lettuce species, but minimal differences in rubber percentages in whole plants. None of the three reports provided information about the purity or composition of the latex the molecular weights of the rubber in the latex, or the condition of the tissues previous to extraction.

In the present study, we examined *L. serriola* (L.) and *L. sativa* (L.) cv. Salinas for presence and quality of rubber in the latex. Our objective was to determine characteristics of natural rubber in these two lettuce accessions, to assess lettuce as a model for rubber biosynthesis. We demonstrate that both species produce latex containing desirable high molecular weight (>1 million g/mol) *cis*-1,4-polyisoprene with very narrow polydispersity that should lead to excellent product characteristics after vulcanization. We show that rubber particles purified from the latex are enzymatically active and that the rubber transferase activity is sensitive to allylic diphosphate initiator concentration. For a few rubber parameters, including molecular weight, we found variation between samples from the two lettuce species, suggesting lettuce provides the opportunity to dissect the genetic and molecular mechanisms underlying natural rubber biosynthesis.

2. Results and discussion

Latex was present in cambial regions throughout the stem and leaf veins upon bolting in *L. sativa* cv. Salinas and *L. serriola*. This is consistent with Kekwick (2001), who mentioned the presence of rubber-containing articulated laticiferous cells accompanying vascular bundles in *Lactuceae*. *Lactuca sativa*, in our greenhouse propagation, had a prolonged rosette stage during which latex was not abundant in leaf veins. After a simple extraction and washing process, the washed latex contained 54% dichloromethane-soluble material (rubber) in *L. serriola* and 47% in *L. sativa*, whereas the remaining material in both species consisted of acetone-soluble material (Table 1). The molecular structure of the major polyisoprene in the dichloromethane-soluble portion of the latex was determined by ^{13}C NMR spectroscopy. In both species, the methyl group had a ^{13}C NMR chemical shift of 23.6 ppm characteristic of *cis*-polyisoprene rubber (Fig. 1), and no evidence of a 16.0 ppm signal, which is indicative of *trans*-polyisoprene gutta percha (Duch and Grant, 1970). Thus, approximately half of washed latex was comprised of rubber.

The acetone-soluble resin material was analyzed by a reversed phase LC–MS, and the three main constituents in both species were lactucopicrin, lactucin-15-oxalate, and 15-(4-hydroxyphenylacetyl)-lactucin-8-sulfate guaianolide sesquiterpene lactones (SLs; Fig. 2). These SLs were associated with rubber particles through the washes, and all three SLs were detected previously in *L. sativa* whole latex (Sessa et al., 2000). A previous report found *L. canadensis* and *L. scariola* to contain rubber between 1.6% and 2.2% of the latex, while resinous material was 11.4% to 12.9% (Fox, 1913). The lower percentages of rubber in this study may be due to the fact that Fox extracted

Table 1
Amounts of acetone and dichloromethane (CD_2Cl_2) soluble material in washed latex, and the percent of dichloromethane amount (rubber)

	Acetone soluble (mg)	STD (mg)	CD_2Cl_2 soluble (mg)	STD (mg)	% CD_2Cl_2 soluble (%)
Lactuca serriola	4.93	2.90	5.87	3.64	54
Lactuca sativa	2.95	0.62	2.6	0.56	47

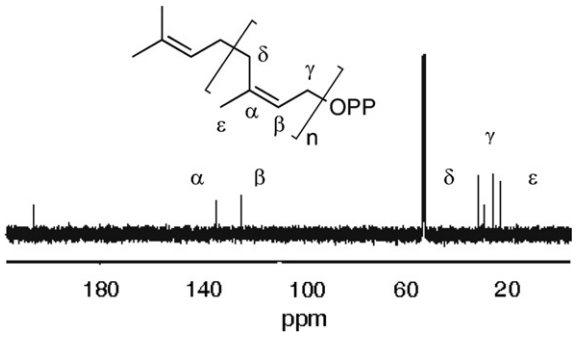

Fig. 1. ^{13}C NMR spectra samples of acetone washed latex isolated from *Lactuca serriola* and dissolved in dichloromethane. The spectra show *cis*-1,4-polyisoprene rubber and no traces of *trans*-polyisoprene gutta. *Lactuca sativa* cv. Salinas had the same spectra.

from whole latex rather than washed latex. The higher percentage of resinous material relative to rubber in the previous study suggests that resinous materials were removed in the washing process that we used.

The ability to process rubber into a high performance product with abrasion resistance and tensile strength is correlated with high molecular weight of the initial *cis*-polyisoprene chain (Swanson et al., 1979). The molecular weights of rubber were estimated at the flowering stage in both species and their extant recombinant inbred line (RIL) population (Argyris et al., 2005). Rubber samples from both species had average molecular weights of over 1,000,000 g/mol, such that the polyisoprene produced by both species is likely of high quality. Additionally, the lettuce molecular weight distribution was similar to that of *H. brasiliensis* rubber (Swanson et al., 1979; Cornish et al., 1993). The molecular weights observed in the *L. sativa* and *L. serriola* samples were not significantly different from each other (Table 2); which is consistent with the two species being con-specific (Kesseli et al., 1991). We have found no previous reports of rubber molecular weights in *Lactuca* species, and relatively few species across the plant kingdom are known to consistently produce rubber with molecular weights above 1,000,000 g/mol (Swanson et al., 1979; Bowers, 1990). In this study, transgressive molecular weight values were detected in the majority of the RIL population, with the parental values less than the progeny mean or median (Fig. 3). This may be the result of genetic, developmental, or environmental influences on molecular weight and suggests the potential for optimizing the genetic, physiological and environmental conditions for the synthesis of higher molecular weight rubber in these species.

Table 2
Mean molecular weight (M_w), polydispersity (M_w/M_n), root mean square (r.m.s.) slopes, and radius (Rz) of *Lactuca sativa* and *Lactuca serriola* rubber samples

	M_w (g/mol)	M_w/M_n	r.m.s.	Rz
Lactuca serriola	1,382,833	1.15	0.433	79.51
Lactuca sativa	1,269,020	1.13	0.362	69.01
	ns	ns	$P < 0.05$[a]	$P < 0.01$

[a] Significant differences between species based on ANOVA.

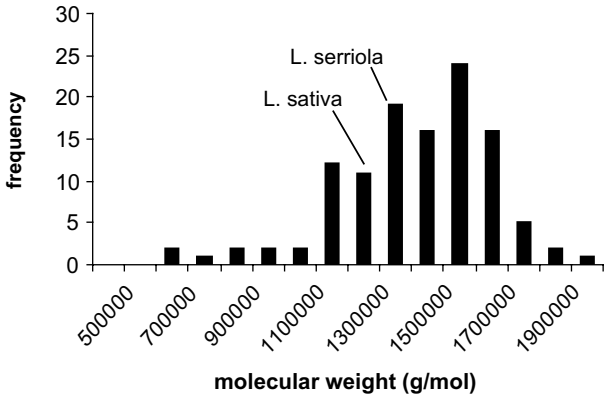

Fig. 3. Rubber molecular weight histogram of 116 recombinant inbred lines derived from *Lactuca sativa* × *Lactuca serriola*.

Polydispersity values were estimated by dividing the weight-average molecular weight (M_w) by the number-average molecular mass (M_n). Higher ratios suggest the presence of non-uniform molecular weight distribution or a mixture of small and large rubber molecules (Wood and Cornish, 2000). For both lettuce species, the values were approximately 1.1, indicating that virtually all the rubber analyzed fell into a narrow molecular weight range (Table 2). It is generally believed that if other structural factors remain constant, lower polydispersity indices lead to desirable properties such as higher impact strength, tensile strength, softening point, etc. (Agrawal et al., 2006). Interestingly, for *H. brasiliensis* rubber, the polydispersity

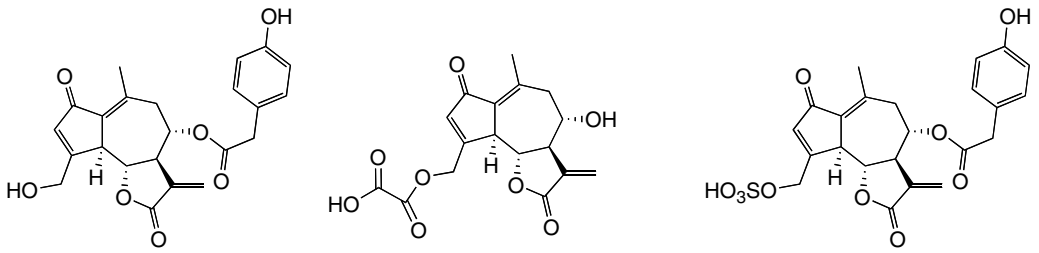

Fig. 2. Sesquiterpene lactones isolated from *Lactuca serriola* and *Lactuca sativa* acetone-soluble extracts.

ranges from 2 to 11 and increases with the age of the tree (Kovuttikulrangsie and Sakdapipanich, 2004).

Rubber is compartmentalized inside microscopic particles, and we estimated the distribution of sizes of rubber particles in both whole and washed *L. serriola* latex (Fig. 4). The average sizes in both whole latex and washed *L. serriola* latex were approximately 5 μm. However, the washed latex also showed a marked increase in the 2 μm range indicating the presence of smaller sized rubber particles that were more apparent upon purification. On average, the particle size distributions of the *L. serriola* were larger than size distributions of *H. brasiliensis* and *P. argentatum*, but similar to *Ficus elastica* (Cornish et al., 1993; Cornish and Brichta, 2002). Rubber particles have been separated previously into categories in *H. brasiliensis* and several *Ficus* species (Singh et al., 2003), of which *F. benghalensis* was most similar to the bimodal distribution of *L. serriola* washed rubber particles in our study.

The rubber polymers purified from both lettuce species exhibited root mean square (r.m.s.) radius slopes of 0.36 and 0.43 (Table 2), indicating a compacted or branched shape (Wyatt, 1993). The *L. sativa* r.m.s. values were significantly lower than the *L. serriola* values indicating that the rubber molecules in the *L. sativa* sample were more tightly coiled and/or branched than the rubber molecules from *L. serriola*. Molecular radii values were also significantly smaller in *L. sativa* than in *L. serriola* samples (Table 2).

Rubber particle-bound rubber transferase enzymes in the latex suspension catalyze the formation of long *cis*-1,4-polyisoprene polymers from sequential additions of isopentenyl diphosphate (IPP) onto an allylic diphosphate initiator molecule (Scholte and Vederas, 2006; Cornish, 2001b). Previous studies have identified farnesyl diphosphate (FPP) as an initiator in several different species (Cornish, 2001b), and this was tested as the initiator in both lettuce species. Samples from anthesis *L. sativa* and *L. serriola* latex catalyzed the formation of rubber polymers from labeled IPP across a range of labeled FPP concentrations (Fig. 5). The kinetic characteristics of the rubber transferase differed between the two, and FPP appeared to become inhibitory in both *Lactuca* species at much lower concentrations than was observed in *H. brasiliensis*, *P. argentatum*, and *F. elastica* (Cornish et al., 2000; Scott et al., 2003). Molecular weights of rubbers in other species have been inversely correlated with *in vitro* FPP concentration, but positively correlated with *in vitro* IPP concentration, at least when FPP was limited (Cornish et al., 2000). The higher rubber transferase activity at lower FPP concentrations in lettuce generally agrees with the previous data in other species, and would suggest that increasing rubber content would likely be reached through increasing the concentration of IPP rather than FPP.

Although biochemical properties of rubber biosynthesis have been reported in other rubber-producing plants (Mooibroek and Cornish, 2000; Cornish, 2001a), no cultivated rubber crops have the geographic distribution, rapid growth and latex accumulation, and genetic resources available to lettuce (Zohary, 1991; Argyris et al., 2005; Wroblewski et al., 2005; http://compgenomics.ucdavis.edu). We show that latex samples from both lettuce accessions contained high molecular weight *cis*-1,4-polyisoprene with a narrow polydispersity range, and exhibited rubber transferase activity. The RIL population resulting from the interspecific cross between the two lettuce accessions showed evidence of transgressive segregation for molecular weights. Variation between the two accessions was detected in r.m.s. slopes, molecule radii, and rubber transferase enzyme activity. The variation present in

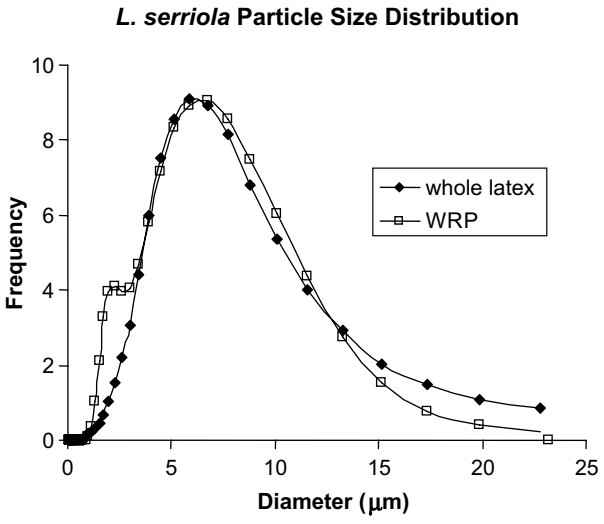

Fig. 4. Particle size distribution of whole latex and washed rubber particles (WRP) from *Lactuca serriola*.

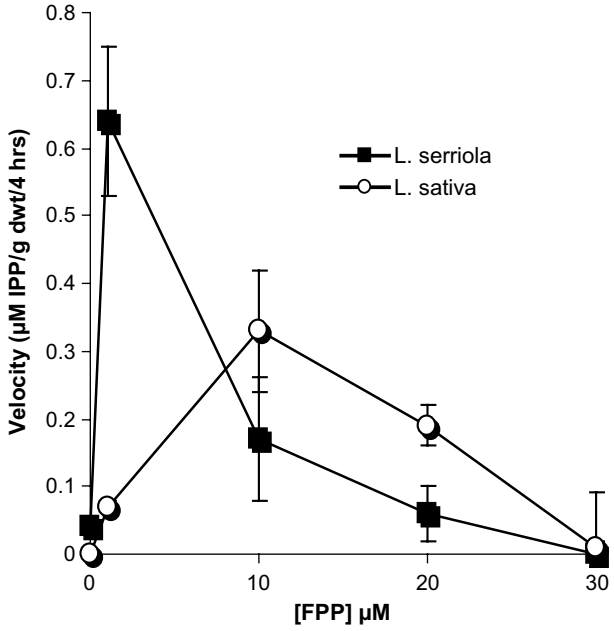

Fig. 5. Rubber transferase activity at 25 °C for 4 h of *Lactuca serriola* and *Lactuca sativa*: with different concentrations of FPP measured by incorporation of 5 mM [^{14}C] IPP.

rubber parameters between the two lettuce accessions in this study, combined with the molecular tools available for lettuce genetics, present lettuce as a tractable experimental system for understanding the genetic and biochemical mechanisms underlying natural rubber biosynthesis. However, it is difficult to predict if lettuce has potential as a domestic rubber source; considerable plant breeding would be needed to modify weedy plant types to test this possibility.

3. Experimental

3.1. Plant materials and latex preparation

Lactuca sativa cv. Salinas and *L. serriola* seed originated from UC96US23 and UG92G488 accession numbers, respectively. Seeds were grown under 14:0 L:D, 75 °C:65 °C day:night temperatures, in Sunshine Mix A soil (Sungro, Vancouver, BC), under standard greenhouse conditions in Corvallis, OR. In addition, for molecular weight estimations, field grown materials of the same two accessions, and 116 F_8 recombinant inbred lines derived from them, were sampled at anthesis from Spence Ranch, Salinas, CA, during February and March of 2003.

Latex was tapped from lettuce stems with a scalpel and dropped in ice-cold wash buffer containing 100 mM Tris–HCl, 5 mM MgCl, 5 mM DTT, 500 mM sucrose, 35% glycerol, and 0.4 mM Pefabloc protease inhibitor (Sigma–Aldrich, St. Louis, MO). The samples were then centrifuged at 8000 rcf at 4 °C, and the latex was removed to a new container of cold wash buffer. This wash was repeated twice more and samples were stored at −20 °C in a final wash buffer without sucrose and glycerol.

3.2. ^{13}C NMR and HPLC/MS

Washed latex, suspended in buffer, was filtered using a centrifugal filtration device (Ultrafree-MC, 0.22 mm, Millipore, Bedford, MA) at 9000 rcf for 10 min to collect solid. Filtered solid was extracted twice with acetone and resulting extracts were combined. The residue was then extracted twice with CH_2CL_2 and combined. The percentage of rubber in the plants was determined by weighing the residues from the dichloromethane extraction, and the percentage of resin in the latex was determined by weighing the residue from the acetone extraction. Both field and greenhouse grown lettuce were tested and included in the means and standard deviations.

For latex composition, the rubber samples were dissolved in 0.7 mL of dichloromethane (Cambridge Isotope Laboratory, Cambridge MA). NMR spectra were recorded on Inova Varian 400 and 500 MHz instruments (Varian Scientific Instruments, Palo Alto, CA). ^{13}C NMR shifts are reported in parts per million (ppm) relative to CH_2CL_2 δ 53.8 (Duch and Grant, 1970). Material isolated from the acetone washing of the latex was analyzed by liquid chromatography–mass spectrometry (LC/MS) on an Agilent 1100 MSD electrospray single quadrapole instrument (Agilent Technologies, Inc., Palo Alto, CA). Five microliter aliquots of resin material in MeOH–H_2O (5:95) were injected onto a reversed phase (RP) C_{18} column (5 μm, 250 × 4.6 mm) with a solvent gradient of 99:1 (H_2O:MeCN) to 1:99 (H_2O:MeCN) over 30 min at 1 ml min^{-1}.

3.3. Molecular weight and particle size analyses

For molecular weight analyses, washed latex was dried and dissolved in tetrahydrofuran (THF) (prefiltered through a 0.2 μm vacuum filter), concentrated between 0.3 and 2.5 mg/ml, and left overnight. The dissolved samples were shaken vigorously and 1 ml aliquots were filtered through 0.45 μm syringe filters composed of PTFE with glass microfibre 25 mm disposable graded density filters (Millipore, Burlington, MA) into autosampler vials with Silicone/Teflon lined septa. Each sample solution (100 μl) was analyzed using a HPLC/MALLS system consisting of a HP 1100 series autosampler, a pump, a HP1047 refractive index detector (Agilent Technologies, Palo Alto, California), a multiple angle laser light scattering detector containing 18 light scattering detectors with a 632.8 nm wavelength laser (Dawn DSP Laser Photometer, Wyatt Technologies, Santa Barbara, California), a Phenogel 5 μm Linear/Mixed Guard Column (Phenomenex, Torrence, California) and a PLgel 10 μm Mixed-B size exclusion column (Agilent Technologies, Palo Alto, California) maintained at 35 °C (Dayal and Mehta, 1994). Latex rubber polymers were eluted within 17 min by THF (1.0 ml min^{-1}). Astra software (Wyatt Technologies, Santa Barbara, CA) was used to calculate molecular weight (weight-averaged, M_w) and root mean square (r.m.s.) radius moments for each peak, as well as polydispersity (M_w/M_n), a measure of diversity in molecular weight. A general description of the use of light scattering for molecular characterization, calibration of the light scattering detector and equations for calculating the various parameters are provided in a review by Wyatt (1993). Particle size distributions were estimated using a Horiba LA-900 Laser Scattering Particle Size Distribution Analyzer (Horiba Instruments Inc., Irvine, CA) according to the manufacturer's instructions. The particle size output was analyzed with LAM-900 and DISP200 software (Horiba Instruments Inc., Irvine, CA).

3.4. Enzyme activity assays

Enzymatically active washed rubber particles from *L. serriola* and *L. sativa* were purified and stored as previously described for *P. argentatum* (Cornish and Backhaus, 1990). Isopentenyl diphosphate incorporation rates were assayed in purified rubber particles (or washed rubber particles – WRP) using a modification of the method by Mau et al. (2000). The reaction took place in individual wells of a 96-well plate. The wells were siliconized with Sigmacote

(Sigma–Aldrich, Corp., St. Louis, MO) for 2 min, rinsed with deionized water and with EtOH–H_2O (95:5), and dried at room temperature overnight. The reaction volume was 50 μL (1 mM IPP; 20 μM FPP; 100,000 dpm of [^{14}C]IPP; 100 mM Tris–HCl, pH 7.5; 5 mM DTT; 5 mM $MgSO_4$). The reaction was begun by the addition of 0.25 mg WRP of sample into each well, and the filter plate was placed on a ceramic cooling plate (Amershan Biosciences, Piscataway, NJ) equipped with a circulating water bath to control the temperature. After 4 h at 25 °C the reaction was stopped by the addition of 2.5 μL 500 mM EDTA. The filters were washed using the Millipore vacuum EtOH–H_2O (95:5, manifold with 100 μL), EtOH–H_2O (95:5, 3×150 μL), and deionized H_2O and EtOH–H_2O (95:5, 2×150 μL) to each well. Filter plates were oven-dried at 37 °C for 30 min, whereupon the filters were removed from the plate and placed individually into vials with 2.5 mL ScintiVerse BD Cocktail (Fisher Scientific, Fairlawn, NJ). The amount of [^{14}C]IPP was determined by liquid scintillation spectroscopy using Beckman LS6500 (Beckman Coulter, Fullerton, CA).

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