Characterizing steps in phytic acid biosynthesis as targets for manipulating inositol phosphate metabolism in soybean

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Phosphorus in plant seeds is stored primarily as phytate (*myo*-inositol hexa*kis*phosphate), an inositol phosphate compound that is poorly digested by non-ruminant animals. A long-term goal of our research is to alter the phytate content of soybean (*Glycine max* L.) seeds to improve phosphorus utilization in feed and reduce phosphorus excretion in animal manure. Developing feed components with improved phosphorus bioavailability will provide additional tools for managing nutrients associated with animal production and will help prevent entry of agricultural phosphorus into critical watersheds.

Phytic acid biosynthesis has been elucidated for a number of organisms including yeast (*Saccharomyces cerevisiae*), slime mold (*Dictyostelium discoideum*), and aquatic duckweed (*Spirodela polyrhiza*). Synthesis can proceed by at least two possible routes: through a phosphatidylinositol pathway (lipid-dependent) as in yeast, or through a series of sequential phosphorylation steps (lipid-independent) as shown for Dictyostelium and Spirodela. Although individual enzymes have been characterized in several plants, a definitive biosynthetic pathway remains undefined. Taking advantage of a large soybean EST (expressed sequence tag) database, we have used a bioinformatics approach to identify clones encoding candidate enzymes involved in inositol phosphate metabolism.

Based on similarity to sequences from other organisms, we have identified cDNAs for three putative pathway enzymes in soybean, inositol (1,3,4)P_3 5/6-kinase, inositol (1,4,5)P_3 6/3-kinase, and inositol (1,3,4,5,6)P_5 2-kinase, as shown in Table 1. We have cloned full-length sequences for the soybean inositol polyphosphate kinases into pGEX vectors for expression of GST-tagged fusion proteins in *E. coli*. Similarity between the four soybean inositol (1,3,4)P_3 5/6-kinase clones and previously characterized sequences from *Arabidopsis thaliana* and *Zea mays* ranges from 51 to 66% at the nucleotide level.

Table 1. Predicted inositol polyphosphate kinases in soybean.

Predicted Enzyme	Open reading frame (bp)	Number of amino acids	Predicted protein size (kD)
Ins(1,3,4)P_3 5/6-kinase	1,020	339	38.4
	948	315	35.0
	1,065	354	40.1
	1,026	341	38.3
Ins(1,4,5)P_3 6/3-kinase	840	279	30.9
Ins(1,3,4,5,6)P_5 2-kinase	1,371	456	51.2

GST fusion proteins were purified by affinity chromatography (Figures 1A and 1B). Purified proteins for Ins(1,3,4)P_3 5/6 kinase showed the ability to phosphorylate [^{3}H]-Ins(1,3,4)P_3 to [^{3}H]-Ins(1,3,4,5)P_4 as detected by high performance liquid chromatography and illustrated in Figure 1C. Similar analysis of reaction products indicated the addition of a

single phosphate during the 15 minute incubation period. Additional characterization will establish whether these enzymes add a second phosphate group and determine the ratio of addition of phosphates at the 5 and 6 positions of the inositol ring. The GST fusion protein from the putative soybean Ins(1,4,5)P_3 6/3-kinase sequence also showed kinase activity, however, no activity has been demonstrated from the soybean Ins(1,3,4,5,6)P_5 2-kinase fusion protein to date. Expression of the soybean sequences is being examined among different tissues and stages of soybean seed development. Sequences from all three enzyme classes are being used to generate silencing constructs in the pHELLSGATE vector. Knock-outs of the different genes will be performed in embryogenic soybean cultures to determine the effects on inositol phosphate metabolism.

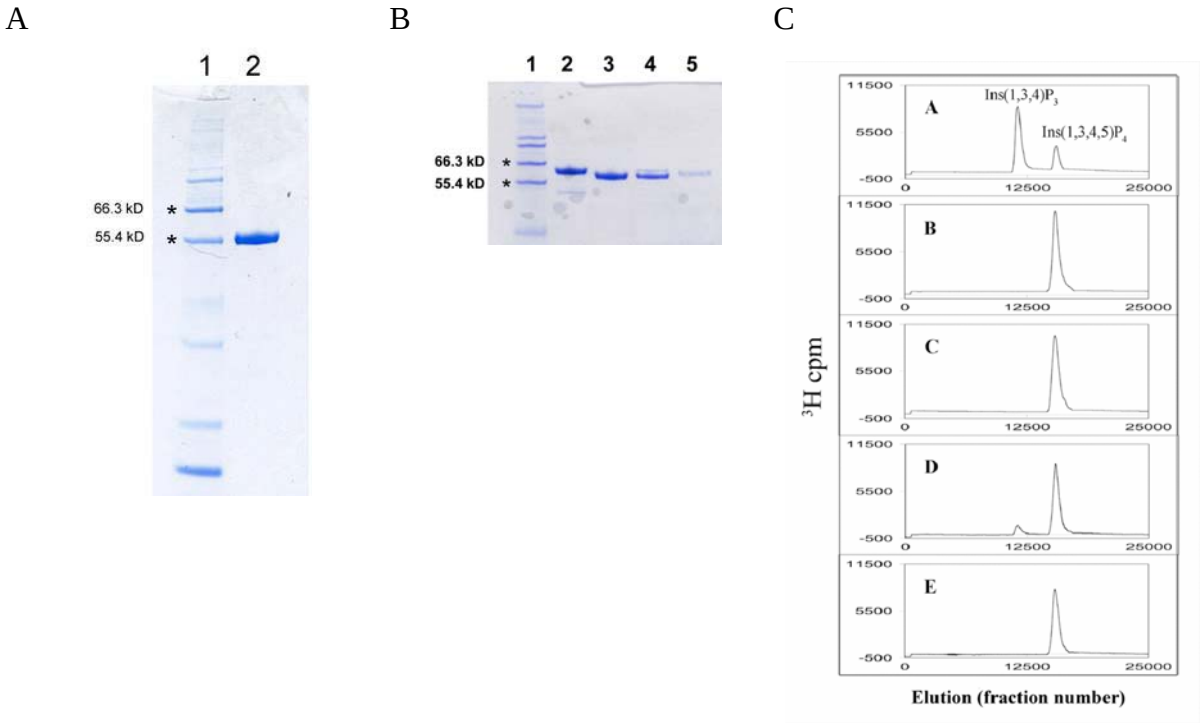

Figure 1. Expression and characterization of GST-kinase fusion proteins. A) GST-tagged fusion protein expressed from a soybean Ins(1,4,5)P_3 6/3-kinase sequence. Lane 1: protein size markers, Lane 2: GST-Ins(1,4,5) P_3 6/3-kinase from soybean. B) GST-tagged fusions proteins expressed from soybean Ins(1,3,4)P_3 5/6-kinase sequences. Lane 1: Protein size markers, Lanes 2-5: four independent GST-Ins(1,3,4)P_3 5/6-kinase fusion proteins. C) HPLC analysis of kinase reaction products. To generate the mixture of substrates shown in panel 1 (control – without adding kinase), [^{3}H]Ins(1,3,4,5)P_4 was incubated with inositol 5'-phosphatase. Panels 2-5 show the HPLC analyses of the products after a 15 min. incubation with a 30 nM substrate mixture of [^{3}H]Ins(1,3,4)P_3 and [^{3}H]Ins(1,3,4,5)P_4, with each of the four GST-Ins(1,3,4)P_3 5/6-kinase fusion proteins. High-performance liquid chromatography was performed with a Waters Spherisorb S5 Sax 4.0 x 125 mm column and a 10 mM to 1.02 M ammonium phosphate gradient.