

Differentially Expressed Forms of 1-L-*myo*-Inositol-1-Phosphate Synthase (EC 5.5.1.4) in *Phaseolus vulgaris**

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We have characterized two distinct polypeptides with 1-L-*myo*-inositol-1-phosphate synthase (MI-1-P synthase) activity that are differentially expressed during development in *Phaseolus vulgaris*. Western analyses, enzyme assays, and partial purification of MI-1-P synthase during embryonic and postembryonic development show that its expression is temporally and spatially regulated.

Developmental Western analyses of soluble proteins detect a small protein, approximately 33 kDa, with MI-1-P synthase activity during the globular stage (stage II) of embryogenesis and in mature roots. Expression of this small protein is also enriched in thylakoidal membranes of fractionated leaf chloroplasts, although Western analyses of total soluble leaf proteins show no cross-reacting material. In contrast, a larger protein, approximately 56 kDa, with MI-1-P synthase activity is present during the cotyledonary phase (stage IV) of embryogenesis in green cotyledons of seedlings and in young roots.

Inositol, a six-carbon cyclitol, is an essential component of eukaryotic cells. Its metabolism is an important concern in many agricultural and clinical disciplines. Inositol phosphates convey signals for a wide variety of hormones, growth factors, and neurotransmitters (1–3). In addition, inositol acts as a precursor for a bewildering array of compounds in plant cells (4).

Inositol 1-phosphate, the immediate precursor of free inositol, is synthesized via an internal cyclization of glucose 6-phosphate (5–8). The enzyme that catalyzes this reaction, MI-1-P synthase,¹ has been purified or partially purified from a number of organisms (9–12). Properties and catalytic mechanisms of MI-1-P synthase are similar in animals, plants, and yeast (14–18). The overall reaction consists of a tightly coupled oxidation and reduction (reviewed in Ref. 18).

Although extensive physiological and biochemical data document the importance of inositol to higher plants (*e.g.* seed germination, membrane formation, cell wall biogenesis, and stress response; reviewed in Ref. 18), less is known of the molecular genetic mechanisms regulating its metabolism. Our objective is to define genetic controls involved in the regulation of inositol synthesis and catabolism in plants. To this end, we

have begun developmental studies of the genes and gene products of the pivotal biosynthetic enzyme, MI-1-P synthase, using the well characterized developmental biology of the green bean, *Phaseolus vulgaris*, and the molecular and classic genetics available in *Arabidopsis thaliana*.

This article reports intriguing findings concerning the temporal and spatial expression of MI-1-P synthase during development of *P. vulgaris*.

EXPERIMENTAL PROCEDURES

Plant Material—Seeds of *P. vulgaris*, Taylor's horticultural variety, were obtained from the Asgrow Seed Co. (Kalamazoo, MI). Plants were grown in soil under standard greenhouse conditions or aseptically in agar medium containing a Murashige and Skoog salt base (with or without inositol) in an environmental chamber maintained at 24 °C with 16-h photoperiods. Embryos were isolated from plants grown in soil and staged according to established criteria (19).

Protein Isolation—Soluble proteins were isolated using 100 mg of tissue crushed in a mortar, cooled by liquid nitrogen, resuspended at 0 °C in 2 ml of buffer, and acetone precipitated (20). A Bio-Rad protein assay, used according to the Bradford method, determined protein concentration. The assay detects the differential color change of a dye in response to various concentrations of protein (21).

Western Blot Analyses—Western blot analyses (22) were performed on proteins separated in SDS-polyacrylamide gels (23) and transferred to nitrocellulose membranes. The yeast polyclonal antibody to MI-1-P synthase (12) was used as a probe.

Partial Purification of MI-1-P Synthase—Partial purification of MI-1-P synthase (12, 13) included the preparation of crude extracts, streptomycin sulfate precipitations, and ammonium sulfate fractionations. Crude extracts (50 g of tissue) were resuspended in buffer (20 mM Tris-HCl and 10 mM NH₄Cl, pH 7.2). Streptomycin sulfate (25%, w/v, solution) was added to a final concentration of 2% (w/v) streptomycin. The supernatant was made 45–70% saturated with solid ammonium sulfate. The 45–70% ammonium sulfate fractions were redissolved in buffer (20 mM Tris-HCl and 10 mM NH₄Cl, pH 7.2) and dialyzed against the same buffer.

Enzyme Assays—Inositol biosynthesis was assayed by the end product method of Chen and Charalampous (8) and the rapid colorimetric method of Barnett *et al.* (24). D-[1-¹⁴C]Glucose 6-phosphate (specific activity, 60.3 mCi/mmol) and [1,2-³H]*myo*-inositol (specific activity, 370–740 GBq/mmol) were obtained from DuPont NEN. Glucose 6-phosphate, bacterial alkaline phosphatase, and phosphate standard were purchased from Sigma.

For the reaction of Hill and Bendall (25), 3 ml of chloroplast (30 µg/A = 1.5) suspension containing 0.1 mM of the dye 2,6-dichlorophenolindophenol (Sigma) was illuminated for 10 min, and the absorbance change was measured at 590 nm. A change of 0.1 was recorded per min. In the presence of the inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea (Sigma), there was no change in absorbance.

Catalase (EC 1.11.1.6) activity was monitored in a quartz cell containing 3.0 ml of 10 mM Tris-Cl (pH 8.5), 0.1 ml of 0.88% H₂O₂ in 100 mM Tris-Cl (pH 8.5), and 0.2 ml of chloroplast extract at 240 nm (26).

Cytochrome *c* oxidase (EC 1.3.9.1) activity was assayed by measuring the initial rate of the aerobic oxidation of ferrocytochrome *c* at 550 nm (27).

Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.13) activity (28) was measured at 366 nm in the direction of NADP⁺ formation (NADPH oxidation), because glyceraldehyde 3-phosphate is stable only as a free acid (pH 5.0) and decomposes rapidly at the optimum pH (7.5–8.5) of the enzyme (28).

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¹ The abbreviations used are: MI-1-P synthase, 1-L-*myo*-inositol-1-phosphate synthase (EC 5.5.1.4); HPLC, high performance liquid chromatography.

TABLE I
Colorimetric determination of MI-1-P synthase activity

Ammonium sulfate-precipitated proteins were assayed for MI-1-P synthase activity using periodate oxidation to measure release of inorganic phosphate from inositol 1-phosphate (24). One unit of MI-1-P synthase is defined as 1 nmol of inositol 1-phosphate produced/h. Total activity = nmol of inositol 1-phosphate produced/h (units $\times 10^{-3}$). Specific activity = nmol of inositol 1-phosphate produced/h per mg of protein (units/mg $\times 10^{-3}$).

Fraction	Protein mg	Total activity units	Specific activity units/mg
Chloroplast			
Envelope (45–70%)	0.013	5	0.385
Stroma (45–70%)	0.022	50	2.273
Thylakoids (45–70%)	0.047	80	1.702
Leaves			
45%	0.560	750	1.334
60%	1.240	800	0.645
70%	0.048	810	1.688
Young roots			
45%	0.160	200	1.250
60%	0.240	900	3.750
70%	0.080	360	4.500
Mature roots			
45%	0.060	80	1.333
60%	0.180	700	3.888
70%	0.140	700	5.000

Paper Chromatography—Inositol synthesized by the end product method using a labeled substrate, D-[1-¹⁴C]glucose 6-phosphate, was identified by paper chromatography (29). Samples were concentrated to a volume of 25 μ l, spotted on Whatman No. 1 paper (10 \times 10 inches), and chromatographed for 12 h in an ascending one-dimensional system with either acetone:water (85:15, v/v) or propanol:pyridine:water (3:1:1, v/v). D-[1-¹⁴C]glucose 6-phosphate and [1,2-³H]myo-inositol were used as standards.

High Performance Liquid Chromatography (HPLC)—Unlabeled inositol synthesized by the end product method was identified using a Waters Dextro-Pak cartridge with a RCM-100 radial compression module and water as the mobile phase. A differential refractometer (R401) and base-line 810 chromatography workstation detected the peaks. All compounds were dissolved in 160 mg of ethanol (internal standard) and 4 ml of water. Twenty microliters of the samples and standards were injected to give about 80% full scale deflection.

Chloroplast Isolation and Fractionation—Intact chloroplasts were isolated by percoll gradient centrifugation (30). Fresh young leaves (100 g) were homogenized in a semifrozen grinding medium (0.35 M sucrose, 25 mM Hepes, and 2 mM EDTA, pH 7.6), strained through eight layers of cheesecloth, washed, and pelleted. The chloroplast pellet was resuspended in buffer (50 mM Hepes-KOH and 0.33 M sorbitol, pH 7.5) and layered on a 10-ml Percoll gradient (2 ml each of 80, 65, 45, 25, and 10% Percoll). The band representing intact chloroplasts (30) was isolated and washed with Hepes-sorbitol buffer. The reaction of Hill and Bendall (25) demonstrated the electron transport activity of the intact Percoll-purified chloroplasts by reducing the dye 2,6-dichlorophenolindophenol at an absorbance decrease of 0.1 per min. The absence of catalase activity (26) and cytochrome *c* oxidase activity (27) precludes any contamination from peroxisomes and mitochondria, respectively. Envelope, thylakoidal, and stromal fractions were prepared from intact Percoll-purified chloroplasts. Cells were lysed in buffer (10 mM Tricine-KOH, 4 mM MgCl₂, and 1 mM phenylmethylsulfonyl fluoride, pH 7.8) and fractionated on a discontinuous sucrose gradient (0.98 and 0.6 M). To check for cross-contamination, each chloroplast fraction (Table I) was tested for glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.13) activity (28). Enzymatic activity was present in the stromal fraction at a specific activity of 4.55. Specific activities for envelope and thylakoidal fractions were negligible.

RESULTS

Developmental Western Analyses—Expression of MI-1-P synthase was analyzed during embryonic and postembryonic development of *P. vulgaris*. Reproductive development in *Phaseolus* can be described in eight stages of approximately equal duration (19). Identification of each stage is based on pod length, seed length, embryo size, and morphology (19). Western blot analyses (Fig. 1A) of these eight stages (lanes 1–8) revealed two cross-reacting proteins. A small protein, approximately 33 kDa, is visible during the globular stage of embryo-

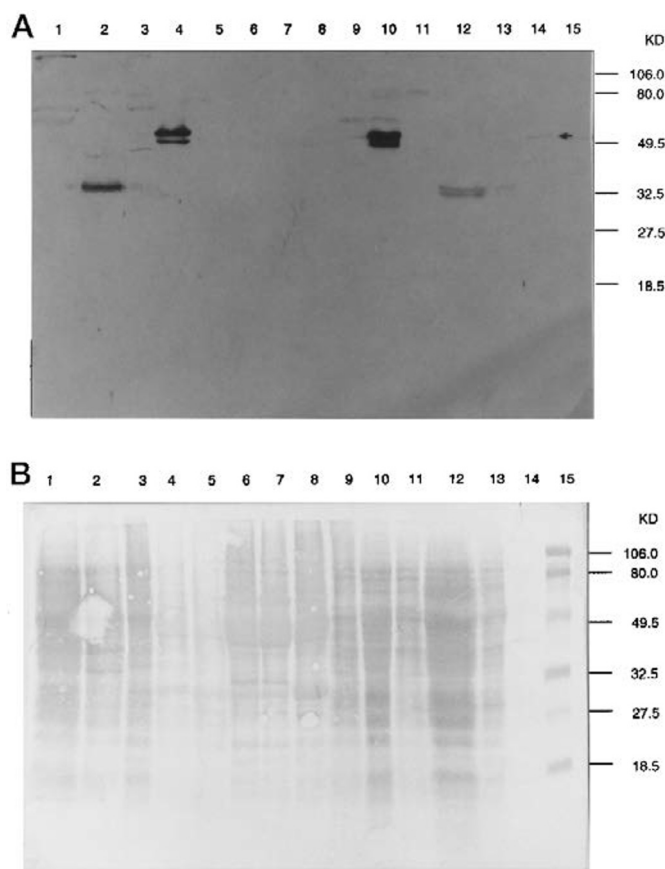


FIG. 1. A, developmental Western blot. Soluble proteins (50 μ g loaded for lanes 1–13) incubated with MI-1-P synthase antibodies were visualized with a nonradioactive chemiluminescence reagent. Lanes 1–8 contain the eight embryonic stages, respectively. Postembryonic organs are contained in lanes 9 and 10 (cotyledons 8 and 18 days after germination, respectively), lane 11 (leaves isolated from soil and aseptic plants 8 and 18 days after germination), lane 12 (mature roots extracted from soil and aseptic plants 18 days after germination), and lane 13 (hook, 2 days after germination). Lane 14 (yeast protein standard) and lane 15 (molecular mass markers) were used to monitor the Western procedure. B, developmental Western blot stained for total protein. To check the efficiency of transfer, the chemiluminescence reagent was removed with rinse buffer, and the nitrocellulose membrane was stained with Amido Black dye, a total protein stain. See A for loading pattern.

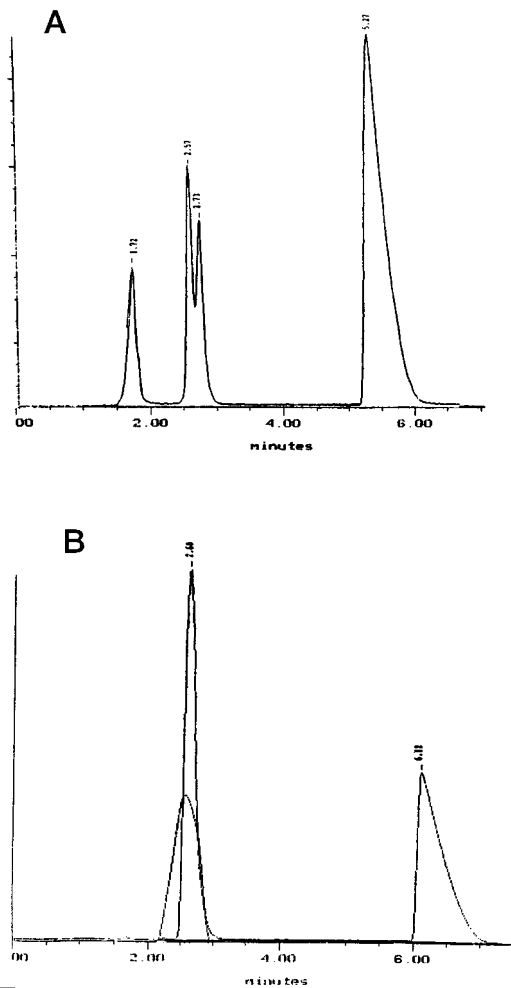


FIG. 2. **HPLC chromatography.** Standards (A) of glucose 6-phosphate (first peak at 1.72 min), inositol (second peak at 2.57 min), glucose (third peak at 2.73 min) and ethanol (fourth peak at 5.27 min) were used as references for retention times. B, overlay of the inositol synthesized in the 70% ammonium sulfate precipitate of leaves (small peak) and the inositol standard (large peak at 2.6 min).

genesis (lane 2). In contrast, a larger protein, 56 kDa (lane 4), is detected during the cotyledonary stage. This protein sometimes appears as a doublet (lane 4). Eighteen days after germination, the 56-kDa protein is visible in cotyledons (lane 10), whereas the 33-kDa protein is found only in mature roots (lane 12). Cross-reacting material was not present in cotyledons (8 days after germination) (lane 9), leaves (lane 11), or hypocotyls (lane 13). A typical total protein profile after Western analysis is presented (Fig. 1B).

Partial Purification of MI-1-P Synthase—To correlate the expression of these proteins with MI-1-P synthase activity, the enzyme was partially purified from young roots, mature roots, and leaves of plants grown aseptically in inositol minus media. We have found that the presence of inositol in plant media represses the expression of the enzyme in *Phaseolus*, as demonstrated with *Arabidopsis* (31). Leaves were used as a negative control, since routine Western analyses of soluble leaf proteins showed no cross-reacting material. Each ammonium sulfate fraction was assayed for MI-1-P synthase activity. As a means of detection, three methods, paper chromatography (8, 29), HPLC, and the rapid colorimetric assay (24) were used, since each has its limits. For HPLC analysis, standards (Fig. 2A) were used as references for retention times. Chromatograms produced from paper and HPLC chromatography showed that inositol was being produced (in varying concentra-

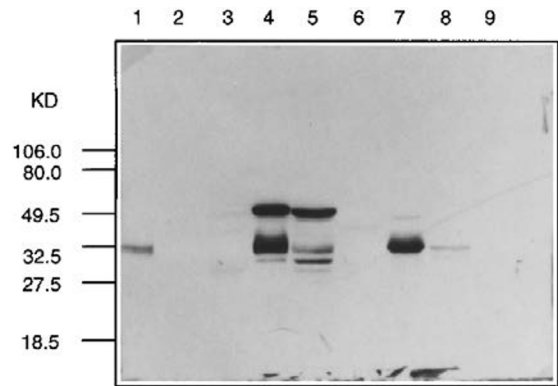


FIG. 3. **Correlation of MI-1-P synthase expression with ammonium sulfate-precipitated activity.** Crude extracts of leaves, young roots (8 days or less after germination), and mature roots (18 days or more after germination) were fractionated with ammonium sulfate at 45, 60, and 70% saturation levels, dialyzed, and assayed for enzymatic activity. Western analysis of precipitates was used to correlate the presence of the enzyme with precipitated activity. Lanes 1-3, leaf extracts; lanes 4-6, young roots; and lanes 7-9, mature roots, at 70, 60, and 45% ammonium sulfate saturations, respectively.

tions) in the ammonium sulfate fractions of all samples. The colorimetric assay (24) (Table I) determined the amount of inositol 1-phosphate produced per hour per mg of protein (specific activity of MI-1-P synthase).

The production of inositol in leaf fractions (Fig. 2B and Table I) was totally unexpected. Subsequent Western analyses of these precipitates (Fig. 3) revealed the presence of the 33-kDa protein in the 70% leaf fraction and the 60 and 70% fractions of young and mature roots. Surprisingly, the 56-kDa protein was also present in the 70 and 60% ammonium sulfate fractions of young roots (Fig. 3).

Chloroplast Isolation and Fractionation—To resolve the ambiguity of the presence of the 33-kDa MI-1-P synthase in 70% ammonium sulfate fractions of leaf proteins when routine Western analyses of soluble leaf proteins showed no cross-reacting material, the literature was searched for a possible subcellular location for the enzyme in leaves. References showed that three investigations have reported MI-1-P synthase activity associated with chloroplasts (32-34). Therefore, we asked if the chloroplast is a subcellular locale for the 33-kDa MI-1-P synthase in *Phaseolus*. Intact chloroplasts were isolated from fresh young leaves in a Percoll-sorbitol gradient and lysed. Centrifugation in a discontinuous sucrose gradient separated lysed chloroplasts into stromal, envelope, and thylakoidal fractions (30). Western blotting (Fig. 4) and enzyme assays of 45-70% ammonium sulfate fractions (Table I) identified the thylakoids as the location for the smaller 33-kDa MI-1-P synthase (Table I). In addition, a cross-reacting higher molecular size protein or protein complex with MI-1-P synthase activity was detected in the envelope and stromal fractions (Fig. 4 and Table I).

DISCUSSION

Our biochemical analyses of the developmental regulation of MI-1-P synthase expression in the green bean establish, for the first time, the foundation needed to genetically dissect the regulation of inositol metabolism in a higher plant. *Phaseolus* provides a useful model system in which to study, at the molecular level, the regulation of inositol biosynthesis during plant growth and development. Embryonic and postgerminative development of organs proceed along a predictable pathway. Furthermore, all stages of development provide ample experimental material for biochemical analyses.

Although we observed no cross-reacting material when sol-

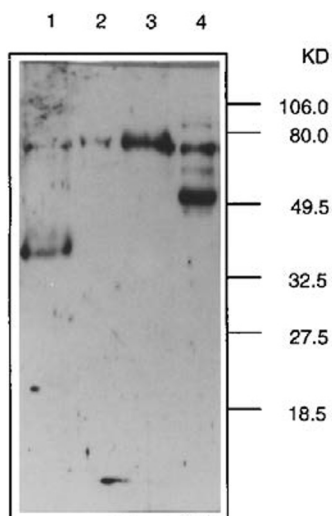


FIG. 4. Immunoblot of thylakoidal (lane 1), envelope (lane 2), and stromal (lane 3) fractions separated in a discontinuous sucrose gradient. Lane 4 contains embryonic (stage IV) protein. Each lane contains 30 μ g of protein.

uble leaf proteins were analyzed by Western blotting, synthase activity in the ammonium sulfate-saturated precipitates of leaf proteins clearly pointed to its presence in leaves. Western analyses of the precipitates showed that the 33-kDa form of the enzyme was indeed located in the leaves. Subsequent isolation and fractionation of *Phaseolus* leaf chloroplasts localized the 33-kDa form of the enzyme to the thylakoids, confirming previous reports of MI-1-P synthase activity associated with these organelles. We also, surprisingly, identified a very large cross-reacting protein or protein complex (approximately 80 kDa) with MI-1-P synthase activity, which localized to the chloroplast envelope and stroma. This protein could represent an active precursor, since most proteins targeted to the thylakoids are synthesized outside of the chloroplasts as larger molecular size precursors and processed to their mature size during transport (35–37). Routine Western analyses and ammonium sulfate fractionation cannot detect this protein.

The developmental profile for the expression of MI-1-P synthase in *Phaseolus* is striking. Expression and associated activity appears at two crucial stages (stages II and IV) during seed maturation. During these stages, two important transient organs, the suspensor (stage II) and cotyledons (stage IV), are formed. Given the postulated functions for the angiosperm suspensor (anchor and/or major route of nutrients into the embryo), it will be of great interest to determine the subcellular localization of MI-1-P synthase (inositol biosynthesis) during this stage of embryogenesis. More importantly, we can now begin to investigate the role of inositol in the developing embryo and/or suspensor. It will also be informative to localize inositol biosynthesis in embryonic and postembryonic cotyledons and to compare the site of inositol synthesis with that of phytic acid (a hexakisphosphoric acid ester of *myo*-inositol that serves as a major phosphate reserve for the seed).

Our working hypothesis based on the data presented and the probability that inositol cannot traverse the plastid membrane (38) is that: 1) MI-1-P synthase (*de novo* biosynthesis of inositol) in *Phaseolus* is localized to plastids in different organs (roots, leaves, suspensor and/or embryo, and cotyledons); and 2) different forms of the enzyme at different stages of development reflect regulatory controls at the transcriptional and translational levels. Support for this hypothesis comes from preliminary Northern analyses, the recent cloning and sequencing of a root cDNA encoding a protein with MI-1-P syn-

thase activity (39), the recent isolation of a *Phaseolus* leaf cDNA clone,² and the presence of an inositol transport system in plants (18).

Developmental studies of inositol biosynthesis in *Arabidopsis* and the isolation of a second *Arabidopsis* cDNA with MI-1-P synthase activity (31, 40, 41) also suggest that transcriptional and translational mechanisms operate to regulate the temporal and spatial expression of MI-1-P synthase in *Arabidopsis*.

These recent findings and the infinite questions and possibilities raised concerning the genetic regulation of inositol biosynthesis in higher plants propel us into the exciting area of chloroplast (plastid) molecular biology. Future experiments, among many, will address the mechanics of translocation of MI-1-P synthase into the chloroplast.

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² M. D. Johnson and X. Wang, unpublished data.