Differentially Expressed Forms of 1-L-myoinositol-1-phosphate synthase (EC 5.5.1.4) in Phaseolus vulgaris*

Margaret Dean Johnson‡ and Xiaohong Wang

From the Department of Biological Sciences, The University of Alabama, Tuscaloosa, Alabama 35487

*This work was supported in part by National Science Foundation Grant MCB-9307092. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡To whom correspondence should be addressed: Dept. of Biological Sciences, The University of Alabama, Box 870344, Tuscaloosa, AL 35487. Tel.: 205-348-1819; Fax: 205-348-1786.

We have characterized two distinct polypeptides with 1-L-myoinositol-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 I) 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 cofactor 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).

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). [1-14C]Glucose 6-phosphate (specific activity, 60.3 mCi/mmol) and [1,2-3H]myoinositol (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 μgA

Calculation (EC 1.1.11.6) 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 1.9.3.1) 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).
Differentially Expressed MI-1-P Synthase in P. vulgaris

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 per mg of protein (units/mg × 10⁻³).

| Fraction       | Protein (mg) | Total activity (units) | Specific activity (units/mg) |
|----------------|--------------|------------------------|-----------------------------|
| Chloroplast    |              |                        |                             |
| Envelope       | 0.013        | 5                      | 0.385                       |
| Stroma         | 0.022        | 50                     | 2.273                       |
| Thylakoids     | 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                       |

**TABLE I**

Colorimetric determination of MI-1-P synthase activity

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.
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 vulgaris 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 path, providing a framework for the foundation needed to genetically dissect the regulation of inositol metabolism in a higher plant. We report here a novel developmental regulation of inositol 1-phosphate synthase (MI-1-P synthase) (45) in Phaseolus vulgaris, a model legume plant. MI-1-P synthase, which catalyzes the biosynthesis of inositol, is a key enzyme in inositol metabolism. 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 vulgaris 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 path. Furthermore, all stages of development provide ample experimental material for biochemical analyses. Although we observed no cross-reacting material when sol-

![Image](78x360 to 278x732)

**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 vulgaris 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-

![Image](331x588 to 538x732)

**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.

![Image](78x360 to 278x732)

**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).

![Image](78x360 to 278x732)

**Differentially Expressed MI-1-P Synthase in P. vulgaris**

![Image](78x360 to 278x732)

A

B

![Image](78x360 to 278x732)

**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).

**Differentially Expressed MI-1-P Synthase in P. vulgaris**

![Image](78x360 to 278x732)

A

B

**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 vulgaris 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-

![Image](78x360 to 278x732)

**Differentially Expressed MI-1-P Synthase in P. vulgaris**

![Image](78x360 to 278x732)

A

B

**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 vulgaris 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-

![Image](78x360 to 278x732)

**Differentially Expressed MI-1-P Synthase in P. vulgaris**

![Image](78x360 to 278x732)

A

B

**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 vulgaris 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-

![Image](78x360 to 278x732)

**Differentially Expressed MI-1-P Synthase in P. vulgaris**

![Image](78x360 to 278x732)

A

B

**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 vulgaris 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-

![Image](78x360 to 278x732)

**Differentially Expressed MI-1-P Synthase in P. vulgaris**

![Image](78x360 to 278x732)

A

B

**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 vulgaris 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-
Differentially Expressed MI-1-P Synthase in P. vulgaris

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 μg 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 organelles, 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 synthase activity (39), the recent isolation of a Phaseolus leaf cDNA clone, 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.

Acknowledgment—We thank Dr. Susan Henry for the yeast antibody.

REFERENCES

1. Hokin, L. E. (1985) Annu. Rev. Biochem. 54, 205–235
2. Berridge, M. J., and Irvine, R. F. (1989) Nature 341, 197–205
3. Berridge, M. J. (1993) Nature 363, 315–325
4. Drobak, B. K. (1993) Biochem. J. 288, 697–712
5. Eisenberg, F., and Bolden, A. (1962) Biochem. Biophys. Res. Commun. 12, 72–77
6. Loewus, F., and Kelly, S. (1962) Biochem. Biophys. Res. Commun. 7, 204–208
7. Eisenberg, F. J., Bolden, A. H., and Loewus, F. A. (1964) Biochem. Biophys. Res. Commun. 14, 439–444
8. Chen, I. W., and Challaampavanam, F. C. (1966) J. Biol. Chem. 241, 2194–2199
9. Loewus, M. W., and Loewus, F. A. (1971) Plant Physiol. (Bethesda) 48, 255–260
10. Ogumeyi, E. O., Pittner, F., and Hoffmann-Ostenhof, O. (1978) Hoppe-Seyler’s Z. Physiol. Chem. 359, 613–616
11. Madsen, T., and Eisenberg, F. (1980). J. Biol. Chem. 255, 8485–8486
12. Donahue, T. F., and Henry, S. A. (1981). J. Biol. Chem. 256, 7077–85
13. Dean-Johnson, M., and Henry, S. A. (1989). J. Biol. Chem. 264, 1274–1283
14. Loewus, M. W., and Loewus, F. A. (1973) Plant Sci. Lett. 1, 65–69
15. Kieley, D. E., and Sherman, W. R. (1975). J. Am. Chem. Soc. 97, 6810–6814
16. Sherman, W. R., Rasheed, A., Mauck, L. A., and Wiecko, J. (1977). J. Biol. Chem. 252, 5697–5676
17. Wang, Y.-H., and Sherman, W. R. (1985) J. Biol. Chem. 260, 11083–11090
18. Loewus, F. A. (1990) Inositol Metabolism in Plants (Boss, W. F., Moret, D. J., and Loewus, F. A., eds) Vol. 9, pp. 13–19, Wiley-Liss, Inc., New York
19. Wabott, V., Clutter, M., and Sussen, I. M. (1972) Phytochemistry 11, 59–68
20. Colas Des Francs, C., Thiellement, H., and De Vienne, D. (1985) Plant Physiol. (Bethesda) 78, 178–182
21. Bradford, M. M. (1976) Anal. Chem. 72, 248–254
22. Towbin, H., Staehelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4350–4354
23. Laemmli, U. K. (1970) Nature 227, 680–685
24. Barnett, J. E. G., Brice, R. E., and Corina, D. L. (1970) Biochem. J. 119, 183–186
25. Hill, R., and Bendall, F. (1960) Nature 186, 136–137
26. Luck, H. (1962) Catalase: Methods of Enzymatic Analysis (Bergmeyer, H. U., ed) pp. 885–894, Academic Press, New York
27. Smith, J. K. (1973) Biochim. Biophys. Acta 321, 156–164
28. Gerff, R. (1982) Methods in Chloroplast Molecular Biology (Edelman, M., Hallick, R. B., and Chua, N. H., eds) pp. 683–694, Elsevier Biomedical Press, New York
29. Trevathan W, Proctor D, and Harrison, J. (1950) Nature 166, 444–445
30. Robinson, C., and Bernet, L. K. (1988) Isolation and Analysis of Chloroplasts: Plant Molecular Biology-A Practical Approach (Shaw, C. H., ed) pp. 1–34, IRL Press, Washington, D.C., Elsevier, Amsterdam
31. Johnson, M. D., and Sussex, I. M. (1995) Plant Physiol. (Bethesda) 107, 613–619
32. Imhoff, V., and Boudor, R. (1973) Phytochemistry 12, 331–336
33. Adhikari, J., Majumder, A. L., Braduri, T. J., DasGupta, S., and Majumder, A. L. (1987) Plant Physiol. (Bethesda) 85, 611–614
34. Wang, M. W., Schif, J. A., and Loewus, F. A. (1986) Acta Bot. Neerl. 35, 347–350
35. Vlietan, P. V., Doner, E. R., and Dunsmuir, P. (1988) J. Biol. Chem. 263, 15000–15007
36. Lampka, G. K. (1988) J. Biol. Chem. 263, 14996–14999
37. Cai, D., Herbrann, R. G., and Klisog, R. B. (1993) Plant J. 3, 833–892
38. Wang, C. T., and Noble, P. S. (1971) Biochim. Biophys. Acta 241, 200–212
39. Wang, X., and Johnson, M. D. (1995) Plant Physiol. (Bethesda) 100, 136
40. Johnson, M. D., and Burk, D. H. (1995) Plant Physiol. (Bethesda) 109, 721
41. Johnson, M. D. (1994) Plant Physiol. (Bethesda) 105, 1023–1024

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