Plant Physiol. (1995) 107: 613–619

1L-*myo*-Inositol 1-Phosphate Synthase from *Arabidopsis thaliana*[1]

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A recombinant phage containing an *Arabidopsis thaliana* cDNA sequence encoding a protein with 1L-*myo*-inositol 1-phosphate synthase (EC 5.5.1.4) activity has been isolated and used for transcriptional and translational studies. The identification of the recombinant phage relied on the observations that (a) the clone complements a mutation in the structural gene for 1L-*myo*-inositol 1-phosphate synthase in the yeast *Saccharomyces cerevisiae*, (b) the in vitro synthesized polypeptide enzymatically converts glucose 6-phosphate into inositol 1-phosphate, (c) in vitro transcription and translation of this cDNA sequence produces a polypeptide that is recognized by anti-yeast *myo*-inositol 1-phosphate synthase antiserum, and (d) inositol regulates the expression of the corresponding gene in *Arabidopsis*.

The biosynthesis and metabolism of inositol and the inositol phospholipids play a central role in the function of eukaryotic cells. Metabolic products of inositol have been shown to play a vital role in signal transmission for a wide variety of hormones, growth factors, and neurotransmitters (Loewus and Loewus, 1983; Hokin, 1985; Morse et al., 1987; Berridge and Irvine, 1989; Boss, 1989; Berridge, 1993). In addition, the metabolism of inositol plays a unique role in enabling eukaryotic cells to cope with hypertonic conditions (Sacher and Staples, 1985; Nakanishi et al., 1989). Most eukaryotic organisms have the capacity to synthesize MI-1-P from Glc-6-P. Studies of the biosynthetic pathway of inositol were initiated with the preparation of a system capable of forming labeled inositol from labeled Glc (Eisenberg and Bolden, 1962; Loewus and Kelly, 1962; Eisenberg et al., 1964; Chen and Charalampous, 1966). Although the enzyme that catalyzes this reaction, MI-1-P synthase (EC 5.5.1.4) has been purified or partially purified from a number of organisms (Loewus and Loewus, 1971, 1983; Ogunyemi et al., 1978; Maeda and Eisenberg, 1980; Donahue and Henry, 1981), the first report of the isolation and characterization of a plant MI-1-P synthase was made in 1971 by Loewus and Loewus. The properties and catalytic mechanisms of this enzyme are similar in animals, plants, and yeast (Kiely and Sherman, 1975; Sherman et al., 1977; Loewus and Loewus, 1983; Wong and Sherman, 1985; Loewus, 1990). The absolute requirement for NAD^+ with no net gain in NADH suggests that the overall reaction consists of a tightly coupled oxidation and reduction (Loewus, 1990).

The *INO1* locus was identified as the structural gene for MI-1-P synthase in yeast (Donahue and Henry, 1981). The *INO1* gene was isolated by genetic complementation of the *ino* mutant phenotype and sequenced (Klig and Henry, 1984; Johnson and Henry, 1989). The DNA sequence of the *INO1* gene revealed a 553-amino acid open reading frame predicted to encode a protein of 62.8 kD (Johnson and Henry, 1989). The amino acid composition and amino-terminal sequence (first eight amino acids) derived from purified yeast MI-1-P synthase were compared with the protein predicted from the sequence of the open reading frame of the *INO1* gene, confirming that it encodes MI-1-P synthase (Johnson and Henry, 1989). This analysis provided the foundation needed to understand the complex regulatory role of inositol in phospholipid biosynthesis in yeast (Johnson and Henry, 1989). Recently, a cDNA encoding MI-1-P synthase was isolated and shown to be rapidly and spatially up-regulated during an ABA-induced morphogenic response in the aquatic angiosperm *Spirodela polyrrhiza* (Smart and Fleming, 1993). Although the importance of inositol to higher plants (seed germination, membrane formation, cell wall biogenesis, hormone response, stress response, etc.) has been known for many years, less is known of the genetic regulation of its metabolism. Our objective is to isolate and study the genes involved in the regulation of inositol biosynthesis and catabolism in higher plants. To this end, we have focused our attention on the isolation and characterization of the genes and gene products of the biosynthetic enzyme MI-1-P synthase and the catabolic enzyme *myo*-inositol oxygenase (EC 1.13.99.1) from *Arabidopsis thaliana*. In this report we describe the genetic, biochemical, and molecular analyses used to iso-

[1] This work was supported in part by a grant (DCB-8710766) to I.M.S. from the National Science Foundation. M.D.J. was supported in part by an American Cancer Society postdoctoral fellowship and a grant from the National Science Foundation (MCB-9307092).

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Abbreviations: AIS, *Arabidopsis* 1-L-*myo*-inositol 1-phosphate; *INO1*, 1L-*myo*-inositol 1-phosphate synthase structural gene from yeast; MI-1-P, 1L-*myo*-inositol 1-phosphate.

late and study a gene that encodes an *Arabidopsis* MI-1-P synthase.

MATERIALS AND METHODS

Plant Material

Wild-type seeds of *Arabidopsis thaliana* (L.) Heynh (strain Columbia), kindly provided by Dr. David W. Meinke (Oklahoma State University), were grown according methods reported previously (Meinke and Sussex, 1979). Typically, for seed production, plants were grown in 3-inch round plastic pots containing a 12:3:1 (v/v) mixture of coarse vermiculite:potting soil:sterilized sand. For aseptically grown plants, seeds were washed in a screw-capped tube containing 5 mL of 30% (v/v) bleach and 1 μL/mL of 20% Triton X-100 and plated on plant agar medium. Plant agar medium contained Murashige and Skoog salt base (Sigma) (one package per liter), 10 mL/L 100× stock organics (0.05 g of nicotinic acid, 0.05 g of pyridoxine-HCl, 0.05 g of thiamine-HCl, 0.2 g of Gly), 30 g/L Suc, and 7 g/L agar. The pH was adjusted to 5.7 to 5.8 with 1 N KOH. *A. thaliana* seedlings were collected and frozen in liquid nitrogen after 1 to 2 weeks of growth in an environmental chamber maintained at 24°C on a 16-h light/8-h dark (LD) cycle.

Complementation of Yeast

Saccharomyces cerevisiae inositol mutant strain *ino1–13, trp1, his3, leu2, ura3* was a gift from Dr. Susan Henry (Carnegie Mellon University, Pittsburgh, PA). A yeast transformation protocol (Burgers and Percival, 1987) was used to transform the inositol mutant strain with the plasmid portion of an *Arabidopsis* cDNA expression library (λ Yes-R) kindly provided by Dr. Ronald W. Davis (Stanford University School of Medicine, Stanford, CA). The λ Yes vector is capable of replicating as a λ phage, a plasmid lysogen in *Escherichia coli*, or as a centromere plasmid in yeast (Elledge et al., 1991). Yeast complementation analysis (Johnston, 1988) and media for growth of yeast *ino1* strains have been described (Culbertson et al., 1976).

Yeast Plasmid Isolation

Autonomous plasmids were rescued from yeast using the protocol of Kenneth and Kassir (1992). Plasmid DNA was isolated from a 1.5-mL culture grown overnight under selective conditions. The DNA pellet was resuspended in 20 μL of water and 10 μL was used to transform the *E. coli* XL-Blue strain (Stratagene) according to the procedure of Chung et al. (1989).

Subcloning

In the multifunctional λ Yes vector, cDNAs are inserted into an *Xho*I site (Elledge et al., 1991). The inserts from the cDNAs were isolated by digestion with *Xho*I and subcloned into the *Xho*I site of pBluescript II SK vector (Stratagene). The bacterial strain XL-Blue was used for all bacterial transformations and subsequent isolation of plasmids for further analysis.

Protein Isolation

Proteins were extracted from *Arabidopsis* seedlings grown aseptically in Petri dishes on plant agar medium without inositol and with various concentrations of inositol. One hundred milligrams of tissue were crushed in a mortar cooled by liquid nitrogen and resuspended at 0°C in 2 mL of buffer (Colas Des Francs et al., 1985). Protein concentration was determined using a Bio-Rad protein assay.

Western Blot Analysis

Western blot analysis (Towbin et al., 1979) was performed on proteins separated in SDS polyacrylamide gels (Laemmli, 1970) and transferred to nitrocellulose membranes. The yeast polyclonal antibody to MI-1-P synthase (Donahue and Henry, 1981) was used as a probe.

Southern Analysis

Southern analysis (Southern, 1975) was used to locate the cDNA sequence within the *Arabidopsis* genome. Genomic DNA was isolated from whole plants as described by Jofuku and Goldberg (1988). *Arabidopsis* genomic restriction fragments were separated in 0.7% agarose gels according to the method of Sambrook et al. (1989).

Northern Analysis

For northern analysis (Thomas, 1980), total RNA was extracted as described by Cox and Goldberg (1988), subjected to two cycles of oligo(dT)-cellulose chromatography to isolate poly(A)$^+$ RNA (Cox and Goldberg, 1988), and subjected to electrophoresis in 1% formaldehyde gels.

Probes

High specific activity (10^8 cpm/μg) radiolabeled RNA transcripts were synthesized in vitro by substituting [^{32}P]CTP or [^{32}P]UTP for cold CTP or UTP in a standard transcription protocol (Stratagene). Unincorporated nucleotides and template DNA were removed by size exclusion chromatography through a small Sephadex G-50 column in 10 mM Tris-Cl, pH 7.5, and 0.1% SDS.

In Vitro Transcription

Intact RNA transcripts were synthesized and capped using an mRNA-capping kit (Stratagene) and protocol. The AIS clone was digested with an appropriate restriction enzyme for utilization of the T7 promoter (Fig. 1). After digestion, the DNA was treated with proteinase K, extracted with phenol/chloroform, and ethanol precipitated. After 60 min of incubation of the DNA template and Stratagene's mCAP reagents at 37°C, the capped RNA was separated from the DNA template by incubation for 5 min more at 37°C with DNase I. The RNA was extracted with phenol/chloroform and ethanol precipitated. The RNA

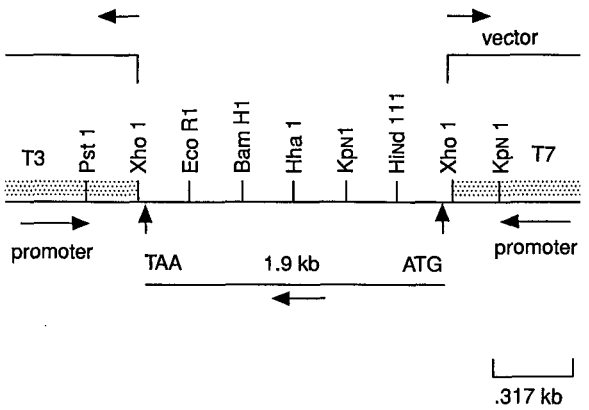

Figure 1. Restriction map of the AIS clone. Restriction sites of the 1.9-kb cDNA subcloned into pBluescript II SK +/− vector were confirmed by DNA sequencing (Johnson, 1994).

pellet was resuspended in 25 μL of an RNase-free Tris-EDTA solution.

In Vitro Translation

The rabbit reticulocyte translation system from Promega and [^{35}S]Met (1200 Ci/mmol, New England Nuclear) was used for cell-free translations. The in vitro transcribed RNA was heated at 67°C for 10 min and then added to the translation mixture. A TCA protein precipitation assay was used to determine the amount of radioactivity incorporated into proteins. The translation reaction mixture (50 μL) was gently vortexed prior to removing a 2-μL aliquot to be analyzed for incorporation (Table I).

Enzyme Assays

The biosynthesis of inositol was assayed by the end product method (Chen and Charalampous, 1966). D-[1-^{14}C]Glc-6-P (specific activity 60.3 mCi/mmol) and [1,2-^{3}H]*myo*-inositol (specific activity 370–740 GBq/mmol) were obtained from New England Nuclear. Glc-6-P and bacterial alkaline phosphatase were purchased from Sigma.

Paper Chromatography

Paper chromatography (Trevelyan, 1950) was used to detect inositol synthesized using the labeled substrate D-[1-^{14}C]Glc-6-P. Samples were concentrated to a volume of 25 μL, spotted on Whatman No. 1 paper (10 × 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). [1-^{14}C]Glc-6-P and [1,2-^{3}H]*myo*-inositol were used as standards.

Table I. *Incorporation of [^{35}S]Met into in vitro translated proteins*

Sample	cpm
Globin mRNA (positive control)	40,803
No RNA (negative control)	390
T 7 transcript	8,033

HPLC Chromatography

The Waters (Milford, MA) Dextro-Pak cartridge with an RCM-100 radial compression module was used to separate inositol and Glc-6-P during HPLC chromatography. Water was used as the mobile phase. Waters differential refractometer (R401) and a baseline 810 chromatography workstation were used to detect the peaks. All compounds were dissolved in 160 mg of ethanol (internal standard) and 4 mL of water (Waters Dextro-Pak Cartridge manual). Solutions (20 μL of standards and products of the enzyme assays) were injected to give about 80% full-scale deflection on the R401 refractive index detector. A baseline 810 computer program was used to analyze and label the curves. The differential refractometer (R401) provided a single-beam optics path that rejected noise and other baseline instabilities.

RESULTS

Isolation of an AIS cDNA Clone

The inositol-requiring strain *ino1–13, trp1, his3, leu2, ura3* was transformed with an *Arabidopsis* cDNA (λ Yes) expression library. The plasmid portion of the λ Yes vector can be looped out of the λ phage by site-specific recombination using the Cre protein and *lox* sites in the vector (Elledge et al., 1991). Depending on its orientation, the cDNA inserted into this vector can either be expressed from the yeast *gal1* promoter or the *E. coli lac* promoter. Transformants were selected sequentially, using uracil and then inositol prototrophy to avoid the possibility of "inositol-less death" (Culbertson et al., 1976). Two thousand uracil prototrophic transformants were screened for inositol prototrophy. The clones were grown on yeast medium without inositol and supplemented with either Gal or Glc. Ten colonies identified as prototrophs for inositol in the presence of Gal only (prototrophy was repressed in the presence of Glc) were retained for further analyses. The plasmid extracted from each yeast colony was amplified in *E. coli* and reisolated. The reisolated plasmid was again used to transform the inositol-requiring yeast strain. Transformants from each plasmid regained uracil and inositol prototrophy simultaneously, confirming that both functions were transferred with the plasmid. Restriction enzyme maps of the 10 plasmids showed that they were all identical (Fig. 1).

Genomic DNA Analysis

To determine the sequences related to the AIS clone in the *Arabidopsis* genome, total genomic DNA was digested with various restriction enzymes and subjected to Southern blot analysis. A radiolabeled single-stranded RNA probe was produced from the 1.9-kb insert (Fig. 1). *Arabidopsis* genomic DNA digested with restriction enzymes *Eco*RI and *Hin*dIII at high stringency resulted in two bands (Fig. 2). At a lower stringency less intense bands could be seen that disappeared when the blot was washed at high stringency (data not shown).

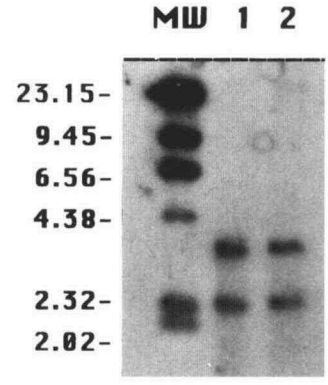

Figure 2. Southern blot analysis of *Arabidopsis* genomic DNA digested with *Eco*RI (lane 1) or *Hin*dIII (lane 2) and probed with the 1.9-kb insert (Fig. 1).

In Vitro Synthesized RNA and Protein

Intact RNA transcripts were generated in vitro for transcriptional and translational studies of the AIS clone. After restriction enzyme digestion to linearize the plasmid, the coding strand (as defined by northern analysis) of the AIS clone was transcribed using the T7 promoter of the pBluescript II KS$^-$ vector (Fig. 1). The transcripts were capped with a 5′ cap structure. The presence of the cap structure has been shown to enhance the translation efficiency of RNA transcripts both by rabbit reticulocyte lysate and by microinjected *Xenopus* oocytes (Berridge and Lane, 1976). SDS polyacrylamide gel (Fig. 3) and western analyses (data not shown) established that the protein derived from the T7 transcript was recognized by the yeast antibody and has a mol wt (approximately 52,000) similar to that of the *Arabidopsis* native protein. Sequence analysis showed that the actual mol wt of the protein was 52,541 (Johnson, 1994). The yeast MI-1-P synthase has a mol wt of 62,842 (Johnson and Henry, 1989).

Enzyme Assays

Having verified that the protein expressed by the AIS clone could indeed cross-react with the yeast antibody, we next tested the expressed protein for its ability to convert ^{14}C-labeled and unlabeled Glc-6-P to inositol 1-P. The enzyme assay was modified in that the reaction was performed in a 250-μL reaction volume (instead of the usual 1 mL) and unlabeled carrier inositol was not added to the reaction tubes after termination of the reaction (Chen and Charalampous, 1966). The reaction product, labeled and unlabeled inositol monophosphate, was converted to free *myo*-inositol by treatment with bacterial alkaline phosphatase. Barium hydroxide was used to hydrolyze Glc. The labeled [^{14}C]inositol resulting from the conversion of [1^{14}C]Glc-6-P was identified by paper chromatography twice, using a different solvent system each time. The first chromatogram (Fig. 4) reflected an acetone and water (85:15) solvent system. The solvent for the second run consisted of propanol:pyridine:water (3:1:1). The R_F values from these runs were comparable to those reported by Culbertson et al. (1976). [1-^{14}C]Glc-6-P and [1,2-^{3}H]inositol were used as standards (Fig. 4). The resulting chromatogram (Fig. 4, lane 2) shows that inositol was only produced in the translation reaction mixture containing protein produced from the T7 transcript of the AIS clone.

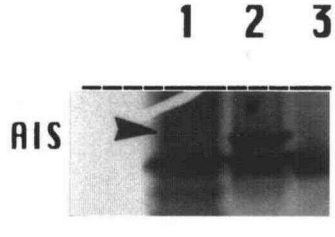

Figure 3. SDS-PAGE of in vitro translated proteins. The cDNA T7 transcript encoded a protein with a mol wt of approximately 52,000 (lane 2) that is recognized by anti-yeast MI-1-P synthase. Lanes 1 and 3 contain the positive (globin mRNA) and negative (no RNA) controls, respectively. All three lanes contain the translation product of an endogenous reticulocyte lysate mRNA.

HPLC Analysis

HPLC provided a more sensitive means of detecting inositol than did paper chromatography. The in vitro synthesized proteins (Table I) were tested for their ability to convert unlabeled Glc-6-P to inositol 1-P. Inositol was produced only by the protein translated from the T7 transcript of the AIS clone (Fig. 5C, peak 2). These results confirmed those obtained using paper chromatography. Although residual Glc was removed when barium hydroxide was added to the reaction mixture, the samples were also run as mixtures of the inositol and Glc standards, since there was little difference between the retention times of inositol (2.450 min) and Glc (2.617 min) using this system.

Inositol Regulates the Expression of AIS

Because the enzymatic activity of MI-1-P synthase has been shown to be repressed by the presence of inositol in plants, animals (Loewus and Loewus, 1983), and yeast (Culbertson et al., 1976), we asked whether the *Arabidopsis* MI-1-P synthase is also regulated in response to inositol.

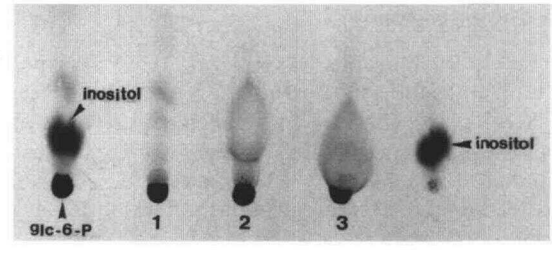

Figure 4. Paper chromatography of the end products generated from enzyme assays of the different translation mixtures. Only the protein produced from the cDNA T7 transcript (lane 2) has MI-1-P synthase activity. Lanes 1 and 3 contain the positive (globin mRNA) and negative (no RNA) controls, respectively. Labeled standards of Glc-6-P and inositol were chromatographed with the samples as controls. One microliter of labeled Glc-6-P was used to define the origin for lanes 1, 2, and 3. The chromatogram was exposed to Kodak (No-Screen) x-ray film for 24 h.

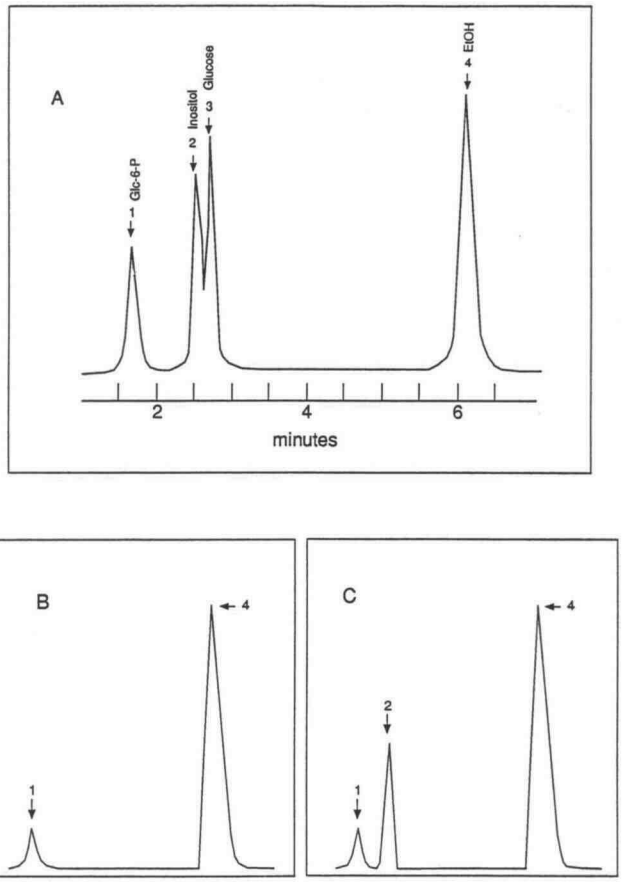

Figure 5. HPLC. A, Standards Glc-6-P (peak 1),inositol (peak 2), Glc (peak 3), and ethanol (peak 4) with retention times (in min) of 1.7, 2.4, 2.6, and 5.7, respectively. B, Chromatogram generated when the control samples, globin and no RNA, is assayed for inositol production. C, Inositol (peak 2), is synthesized only when the protein translated from the cDNA T7 transcript is assayed for MI-1-P synthase activity.

We grew *Arabidopsis* seedlings aseptically in Petri dishes containing plant agar medium with various concentrations of inositol. RNA and proteins were isolated. Northern analysis of the mRNA isolated from these seedlings showed that inositol regulates the expression of its MI-1-P synthase. The isolated *Arabidopsis* cDNA detected a 1.9-kb mRNA from seedlings grown in the absence or low concentrations of inositol (Fig. 6). The 1.9-kb mRNA was not detected in seedlings grown in inositol concentrations of 50 mM or more (Fig. 6). Although northern blot analysis could detect some 1.9-kb message in seedlings grown in 25 mM inositol, western blot analysis could not detect the protein (Fig. 7).

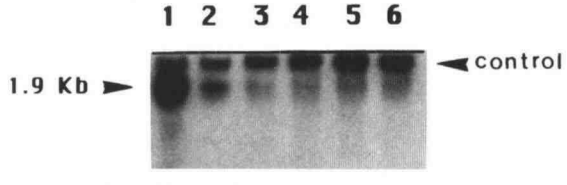

Figure 6. Northern blot analysis. mRNA (10 μg) was isolated from *Arabidopsis* seedlings grown in the absence (lane 1) and presence of increasing concentrations of inositol: 25, 50, 75, 100, and 125 mM (lanes 2–6, respectively). When the AIS clone is used as a probe, a 1.9-kb message is detected in seedlings grown in the absence (lane 1) but not in the presence of inositol concentrations of 50 mM or greater (lanes 3–6). A labeled probe to residual 28S rRNA was used as a loading control (marked control).

DISCUSSION

We began our studies of the regulation of inositol biosynthesis and metabolism by isolating and studying a gene that encodes the biosynthetic enzyme MI-1-P synthase. The enzyme is known to catalyze a complex series of reactions that involve at least three partial reactions (Loewus, 1990). A mechanism based on experimental observations has been proposed (Sherman et al., 1977; Wong and Sherman, 1985).

The identity of an *Arabidopsis* cDNA encoding MI-1-P synthase was established using a combination of genetic, biochemical, and molecular analyses. The gene was isolated by genetic complementation of a yeast *ino1* mutation. In vitro transcription and translation of the gene produced a protein that can convert Glc-6-P to inositol monophosphate. This protein is also recognized by yeast anti-MI-1-P synthase antiserum. The calculated molecular mass of the protein as measured by its migration in SDS polyacrylamide gels is approximately 52.0 kD (Fig. 7). Compared to animal and yeast sources, the reported molecular weights for MI-1-P synthase have been lower for plants (Loewus and Loewus, 1983). Western and northern analyses of proteins and mRNA isolated from *A. thaliana* seedlings grown aseptically in the presence and absence of inositol indicate that inositol has a regulatory role (at the transcriptional level) in the expression of MI-1-P synthase. Expression of the enzyme is repressed by the presence of inositol in concentrations of 50 mM or more. Others have also shown that the expression of MI-1-P synthase in plants (*Acer pseudoplatanus* cell culture) is repressed in the presence of inositol (Loewus and Loewus, 1983). In animal systems, investigators have shown that there is selective hormonal control of inositol biosynthesis in reproductive organs and liver of the male rat (Hasegawa and Eisenberg, 1981). MI-1-P synthase is regulated both in response to exogenous inositol and to unlinked regulatory genes in yeast (Culbert-

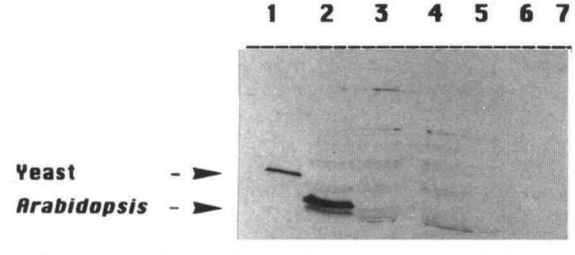

Figure 7. Western blot analysis of the proteins of *Arabidopsis* seedlings grown aseptically on plant agar medium containing various concentrations of inositol (0–125 mM) and probed with yeast anti-MI-1-P synthase. Cross-reacting protein is present only in seedlings grown in less than 25 mM inositol. Lane 1, The 62,842 mol wt yeast MI-1-P synthase (positive control). Lane 2, Protein extracted from *Arabidopsis* seedlings grown in the absence of inositol. Lanes 3 to 7, Proteins isolated from seedlings grown on medium containing 25, 50, 75, 100, or 125 mM inositol, respectively.

son and Henry, 1976; Donahue and Henry, 1981; Greenberg et al., 1982; Loewy and Henry, 1984).

Comparison of the primary structure of the *Arabidopsis* synthase to other proteins found in data banks revealed that the primary structure of MI-1-P synthase, thus far, is highly conserved (Johnson and Henry, 1989; Smart and Fleming, 1993; Johnson, 1994). The primary structure of a *Phaseolus vulgaris* MI-1-P synthase has recently been added to the data banks (M.D. Johnson and X. Wang, unpublished results). It will be of great interest to determine whether the active sites of the enzyme have been evolutionarily conserved.

Studies of the regulation and subcellular localization of MI-1-P synthase during embryonic and postembryonic development in *Arabidopsis* (M.D. Johnson and K. Lackey, unpublished results) and *Phaseolus* (M.D. Johnson and X. Wang, unpublished results) are currently under way. Expression of the enzyme is temporally and spatially regulated. We hope to define, genetically, inositol's role in these developmental processes by isolating and characterizing mutants in *Arabidopsis* that lack a functioning MI-1-P synthase and oxygenase.

Crucial to our studies of the genetic regulation of inositol biosynthesis and metabolism in higher plants, however, is a molecular analysis of the organization of the locus that encodes MI-1-P synthase. We are currently addressing this question.

ACKNOWLEDGMENTS

We wish to thank Dr. Susan Henry for the yeast mutants and antibodies, Dr. Ron Davis for the λ Yes library, Dr. Ed Stephenson and Dr. Janis O'Donnell for critically reading this manuscript, and Dr. Harriett Smith-Somerville for critical analysis of figures.

Received September 22, 1994; accepted October 28, 1994.

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