

VTC4 Is a Bifunctional Enzyme That Affects Myoinositol and Ascorbate Biosynthesis in Plants^{1,2}[W][OA]

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Myoinositol synthesis and catabolism are crucial in many multicellular eukaryotes for the production of phosphatidylinositol signaling molecules, glycerophosphoinositide membrane anchors, cell wall pectic noncellulosic polysaccharides, and several other molecules including ascorbate. Myoinositol monophosphatase (IMP) is a major enzyme required for the synthesis of myoinositol and the breakdown of myoinositol (1,4,5)trisphosphate, a potent second messenger involved in many biological activities. It has been shown that the VTC4 enzyme from kiwifruit (*Actinidia deliciosa*) has similarity to IMP and can hydrolyze L-galactose 1-phosphate (L-Gal 1-P), suggesting that this enzyme may be bifunctional and linked with two potential pathways of plant ascorbate synthesis. We describe here the kinetic comparison of the Arabidopsis (*Arabidopsis thaliana*) recombinant VTC4 with D-myoinositol 3-phosphate (D-Ins 3-P) and L-Gal 1-P. Purified VTC4 has only a small difference in the V_{max}/K_m for L-Gal 1-P as compared with D-Ins 3-P and can utilize other related substrates. Inhibition by either Ca^{2+} or Li^+ , known to disrupt cell signaling, was the same with both L-Gal 1-P and D-Ins 3-P. To determine whether the VTC4 gene impacts myoinositol synthesis in Arabidopsis, we isolated T-DNA knockout lines of VTC4 that exhibit small perturbations in abscisic acid, salt, and cold responses. Analysis of metabolite levels in *vtc4* mutants showed that less myoinositol and ascorbate accumulate in these mutants. Therefore, VTC4 is a bifunctional enzyme that impacts both myoinositol and ascorbate synthesis pathways.

Myoinositol is a six-member carbon ring polyol that is synthesized by both eukaryotes and prokaryotes (for review, see Michell, 2007). In multicellular eukaryotes, myoinositol becomes incorporated into many crucial cellular compounds, including those involved in signal transduction such as phosphatidylinositol phosphates and myoinositol phosphates (InsPs; for review, see Boss et al., 2006), gene expression (InsPs; for review, see Alcazar-Roman and Went, 2007), auxin perception and phosphorus storage (myoinositol hexakisphosphate [InsP₆]; for review, see Raboy and Bowen, 2006; Tan et al., 2007), membrane tethering (glycerophosphoinositide anchors; for review, see Fujita and Jigami, 2007), stress tolerance (ononitol, pinitol; for review, see Taji et al., 2006), and oligosaccharide synthesis (galactinol; for review, see Karner et al., 2004; Fig. 1). Its primary breakdown product, D-GlcUA, is utilized for the synthesis of cell wall pectic noncellu-

losic compounds (for review, see Loewus, 2006) and, in some organisms, ascorbate (for review, see Linster and Van Schaftingen, 2007). Thus, myoinositol synthesis and catabolism affect metabolites involved in many different and critical biochemical pathways.

Although organisms incorporate myoinositol into various compounds, there is only one biosynthetic route to produce myoinositol in what has been referred to as the Loewus pathway (Eisenberg et al., 1964; Chen and Charalampous, 1966; Sherman et al., 1969; Loewus and Loewus, 1980; Loewus et al., 1980). The conversion of Glc-6-P to InsP is catalyzed by the myoinositol phosphate synthase (EC 5.5.1.4; for review, see GhoshDastidar et al., 2006). The product of this reaction can be referred to as either L-myoinositol 1-P or D-myoinositol 3-P (D-Ins 3-P), which are equivalent compounds. The conversion of D-Ins 3-P to free myoinositol is catalyzed by the myoinositol monophosphatase (IMP; EC 3.1.3.25; for review, see Torabinejad and Gillaspay, 2006). We have been interested in the function of IMP in both de novo myoinositol synthesis and during myoinositol second messenger recycling from myoinositol phosphate signaling molecules, such as D-myoinositol 1-P (D-Ins 1-P; Fig. 1). IMP is encoded by multiple genes in plants (e.g. three IMP genes have been examined in tomato [*Solanum lycopersicum*]; Gillaspay et al., 1995). The three different tomato IMPs are highly conserved enzymes that act specifically on monophosphorylated substrates and are inhibited by LiCl (Gillaspay et al., 1995; Berdy et al., 2001). IMP gene expression is developmentally regulated, as is the accumulation of IMP proteins, with maximal levels being present in plant tissues undergoing rapid

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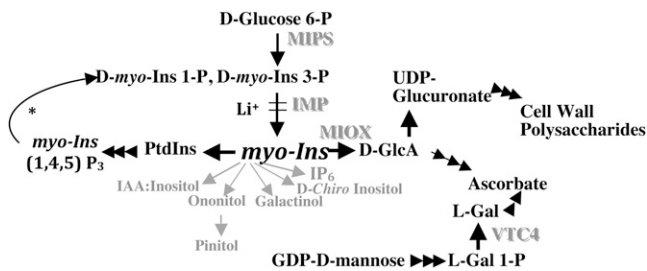


Figure 1. Myoinositol synthesis and metabolism pathway. De novo synthesis of myoinositol (i.e. the Loewus pathway) is catalyzed by myoinositol phosphate synthase (MIPS) and IMP, where its immediate precursor is D-Ins 3-P = L-Ins 1-P. IMP also regenerates myoinositol from the second messenger D-Ins(1,4,5)P₃. Oxidation of inositol by myoinositol oxygenase (MIOX) produces D-GlcUA (D-GlcA), which is a possible entry point into ascorbate synthesis. The major route to ascorbate in plants is the Smirnoff-Wheeler pathway and utilizes GDP-D-Man. VTC4 has homology to the animal IMPs and has been shown to catalyze the conversion of L-Gal 1-P to L-Gal in the Smirnoff-Wheeler pathway. Inositol is also the precursor for the synthesis of several compounds indicated in gray. The asterisk indicates the inositol signaling pathway.

cell divisions, such as seedlings and developing anthers (Gillaspy et al., 1995; Suzuki et al., 2007).

In contrast, *Arabidopsis* (*Arabidopsis thaliana*) contains one potential IMP gene (At3g02870), which was previously identified as functioning in ascorbate synthesis and named VTC4 (Laing et al., 2004; Conklin et al., 2006). Two other genes (At1g31190 and At4g39120) encode proteins that we have classified as IMP-like (IMPL), because of their greater homology to the prokaryotic IMPs, such as the SuhB (Matsuhisa et al., 1995; Chen and Roberts, 2000) and CysQ (Neuwald et al., 1992; Peng and Verma, 1995) proteins. Prokaryotic IMPLs are known to dephosphorylate D-Ins 1-P and other substrates *in vitro*; however, the function of these IMPL proteins is currently unknown (for review, see Roberts, 2006).

Intriguing data suggest that animal IMP is a bifunctional enzyme. The animal IMP hydrolyzes D-Gal 1-P, which is involved in Gal metabolism (Parthasarathy et al., 1997). Furthermore, expression of human IMP can suppress Gal toxicity in yeast (Mehta et al., 1999). Efforts to isolate an L-Gal 1-P phosphatase required for ascorbate synthesis in plants revealed that the kiwi (*Actinidia deliciosa*) and *Arabidopsis* VTC4 can hydrolyze L-Gal 1-P (Laing et al., 2004). This fact prompted the proposal that VTC4 functions mainly to hydrolyze L-Gal 1-P during ascorbate synthesis and that other, unidentified enzymes might be responsible for de novo myoinositol synthesis in plants. This idea is supported by the fact that a *vtc4* loss-of-function mutant contains lower ascorbate levels (Conklin et al., 2006).

Since VTC4 and the IMPLs are the best candidates for enzymes with IMP activity, it is crucial to understand whether these enzymes impact myoinositol synthesis in plants *in vivo*. To determine whether

VTC4 is bifunctional and functions during InsP hydrolysis as well as L-Gal 1-P hydrolysis, we expressed recombinant *Arabidopsis* VTC4 protein and compared the kinetic constants for both D-Ins 3-P and L-Gal 1-P. In contrast to previously reported results, we report here that VTC4 hydrolyzes both substrates well and thus should be considered a bifunctional enzyme. We investigated loss-of-function *vtc4* mutant plants and confirm that these plants contain lower ascorbate levels. We also find reduced myoinositol levels in *vtc4* mutants, supporting a direct role for VTC4 in InsP hydrolysis in plants.

RESULTS

Expression of Recombinant AtVTC4 Protein

To examine whether VTC4 is a bifunctional enzyme, the open reading frame of the *VTC4* gene (At3g02870) was cloned into a plasmid construct containing the T7 viral promoter, an N-terminal polyhistidine (6xHIS) peptide, an Xpress epitope, and an EK cleavage site (pAtIMP). Overexpression of soluble VTC4 protein in *Escherichia coli* strain pREP4/BL21(DE3)* transformed with pAtIMP was facilitated by the coexpression of GroES and GroEL. After cell lysis, the 6xHIS region of the protein bound to the Qiagen nickel-nitrilotriacetic acid agarose column in the presence of phosphate, myoinositol, and Triton X-100 and was eluted with buffer containing 100 mM imidazole. The eluted fraction of VTC4 was greater than 95% pure as observed by 12% SDS-PAGE (Fig. 2), yielding a total of 0.77 mg. By polyacrylamide gel fractionation, the molecular mass of the protein is estimated to be 39.7 kD. Therefore, VTC4 migrates slightly slower than one would expect for a recombinant protein with a predicted molecular mass of 33.1 kD. The eluted fraction was dialyzed in the presence of buffer with 1 mM dithiothreitol to maintain activity and used for biochemical assays.

It has been reported that Mg²⁺ is necessary for maximal activity of IMP and that a pH of 7.5 is optimal (Gumber et al., 1984; Laing et al., 2004; Islas-Flores and Villanueva, 2007). Selected concentrations from 0 to 50 mM MgCl₂ were added to reactions with D-Ins 3-P to determine the optimum MgCl₂ for enzyme activity (Fig. 3A). We conclude that 3 to 4 mM MgCl₂ is the most effective concentration, activating the enzyme to approximately 3-fold higher activity, compared with the reaction without MgCl₂. Additionally, we verified that a pH of 7.5 produced maximal activity (Fig. 3B).

VTC4 has recently been proposed to be an L-Gal 1-P-specific phosphatase (Laing et al., 2004). Since the issue of substrate preference is crucial to understanding myoinositol synthesis in plants, we analyzed the ability of VTC4 to utilize other substrates (Table I). As expected, the substrate derived from D-Ins(1,4,5)P₃ second messenger breakdown, D-Ins 1-P (Fig. 1), is an equally effective substrate for VTC4 as compared

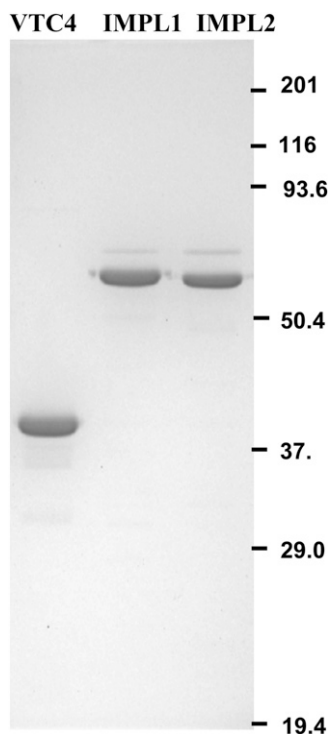


Figure 2. Gel fractionation of purified VTC4 and IMPLs. The indicated purified recombinant proteins (1.5 μ g) were fractionated by 12% SDS-PAGE and stained with Coomassie Brilliant Blue dye. Molecular mass markers, with sizes in kD, are indicated on the right.

with D-Ins 3-P (Table I). The best substrate, however, is L-Gal 1-P, with a 1.6- to 2.4-fold difference in activity between L-Gal 1-P and either InsP. Thus, under our reaction conditions, D-Ins 3-P, D-Ins 1-P, and L-Gal 1-P are all likely to be good substrates for VTC4. β -Glycerophosphate is also a good substrate (52% of the D-Ins 3-P rate of reaction). In contrast, D-Gal 1-P, D-Glc 1-P, D-mannitol 1-P, and adenosine 2'-monophosphate serve less well as substrates (Table I). In addition, Fru 1-P, Fru 1,6-bisP, Glc 6-P, D- α -glycerophosphate, sorbitol 6-P, and myoinositol 2-P are not good substrates for VTC4, as seen by little to no hydrolysis in assays (Table I). Together, these data indicate that VTC4 is a somewhat promiscuous enzyme and that the C1 phosphate position in a six-member ring substrate is important for catalysis, as was noted previously by others (Gumber et al., 1984; Laing et al., 2004; Islas-Flores and Villanueva, 2007). In contrast to what has been reported previously (Laing et al., 2004), we conclude that VTC4 has a minor (1.6- to 2.4-fold) difference in substrate hydrolysis of L-Gal 1-P as compared with D-Ins 3-P or D-Ins 1-P. We also note the difference between VTC4 and human IMP regarding D-Gal 1-P. The human IMP hydrolyzes D-Gal 1-P as effectively as D-Ins 1-P (Parthasarathy et al., 1997), whereas VTC4 appears to be selective for L-Gal 1-P.

Catalytic properties of enzymes are also important factors in substrate preference. In reaction mixtures of

pH 7.5, 4 mM $MgCl_2$, and 1.5 μ g of enzyme, the apparent K_m for D-Ins 3-P was 191 μ M (Fig. 4A) and that for L-Gal 1-P was 107 μ M (Fig. 4B). Substrate or product inhibition of activity was observed with greater than 0.6 to 0.8 mM D-Ins 3-P. The apparent V_{max} for VTC4 with D-Ins 3-P was 4.0 units and that for L-Gal 1-P was 5.4 units. In general, these kinetic parameters are comparable to those found for the *Lilium* IMP (K_m of 78 μ M for D-Ins 3-P; Loewus and Loewus, 1982) and kiwifruit VTC4 (K_m of 150 μ M for L-Gal 1-P and 330 μ M for D-Ins 3-P; Laing et al., 2004; Table II), yet they differ from the previously reported values for the *Synechocystis* (Patra et al., 2007) and barley (*Hordeum vulgare*) enzymes (Fu et al., 2008), which have lower K_m values (Table II). Furthermore, the ratio of V_{max} to K_m provides one measure of discriminating substrate preference, and we found that there is a 1.7-fold difference in the ratios, indicating a small difference between L-Gal 1-P and D-Ins 3-P (Table II).

It was important to determine the inhibition of VTC4 for both substrates by the cations that affect other IMPs. LiCl inhibition of activity with D-Ins 3-P exhibited a linear noncompetitive inhibition, with a K_i of 6.3 mM (data not shown). In addition, the inhibition of activity by LiCl or $CaCl_2$ was similar for either substrate (Fig. 4C). The half-maximal inhibitory concentration (IC_{50}) of VTC4 with $CaCl_2$ as an inhibitor of

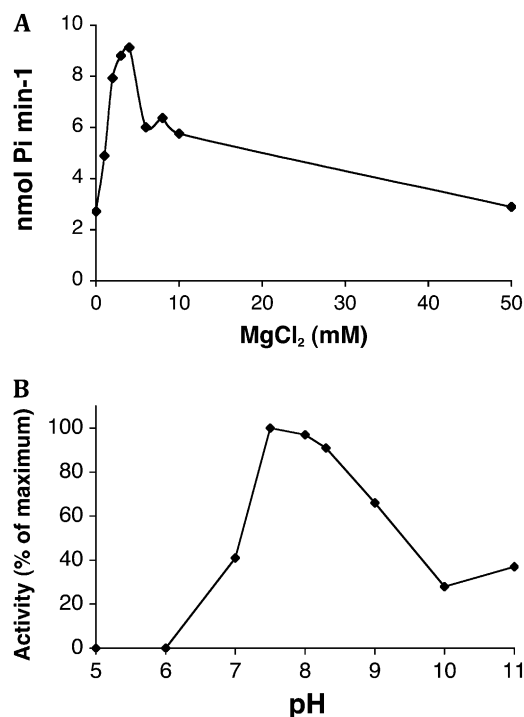


Figure 3. Mg^{2+} and pH dependence of purified VTC4. A, Purified VTC4 (1.5 μ g) was incubated in Tris-Cl (pH 7.5) with 0.5 mM D-Ins 3-P for 10 min with varying concentrations of $MgCl_2$. Enzyme activity was determined by phosphate release. B, As in A, except that pH was varied with Tris-Cl buffers and 4 mM $MgCl_2$ was present in all assays.

Table 1. Substrate preference of VTC4

Substrate ^a	Rate ^b
	%
L-Gal 1-P	166–240
D-Myoinositol 1-monophosphate	100
β -Glycerophosphate (glycerol 2-P)	52
α -D-Glc 1-P	19.3
D-Gal 1-P	16.6
D-Mannitol 1-P	10.5
Adenosine 2'-monophosphate	9.6
D- α -Glycerophosphate (glycerol 3-P)	4.9
D-Fru 1-P	2.3
D-Sorbitol 6-P	1.7
D-Myoinositol 2-monophosphate	0.94
Fru 1,6-bisP	0.30
D-Glc 6-P	0.25

^aConcentrations of substrates were 0.4 mM. ^bEnzyme activity was determined under standard reaction conditions as defined in "Materials and Methods" using the phosphate release assay, 1.5 μ g of enzyme, and the indicated substrate. Reaction rates were compared with the rate of activity with 0.4 mM D-Ins 3-P (3.0 units).

the reaction containing 0.5 mM substrate was 0.08 to 0.1 mM, while the IC_{50} for LiCl was 3.5 to 5 mM. This compares favorably with the inhibition of IMP from soybean (K_i with LiCl of 1 mM; Islas-Flores and Villanueva, 2007), kiwi (K_i with LiCl of 3.7 mM; Laing et al., 2004), tomato (IC_{50} with LiCl of 0.01–0.05 mM; Gillaspay et al., 1995), and mammalian brain (IC_{50} with Ca^{2+} of 0.05–0.1 mM; Parthasarathy et al., 1994). Inhibition was also tested in similar reactions utilizing D-Ins 3-P and L-Gal 1-P with 0.01, 0.03, 0.1, 0.3, 1.0, and 3.0 mM ascorbate. There was no appreciable difference in activity in these assays; thus, we conclude that ascorbate is not an inhibitor of VTC4.

Identification of Loss-of-Function *vtc4* Mutants

The bifunctionality of VTC4 observed in vitro suggests that both myoinositol and ascorbate synthesis could be affected by this gene. To determine whether both pathways are affected, we isolated three independent T-DNA insertion mutants. Seeds for *vtc4-2* (SAIL_105_D11), *vtc4-3* (SAIL_843_G10), and *vtc4-4* (SALK_077222) were identified from the SALK T-DNA mutant database and were verified by sequencing of amplified PCR products. The *vtc4-2* mutant contains two tandem T-DNA insertions occurring 1,341 nucleotides from the start site of translation. The *vtc4-3* and *vtc4-4* mutants contain T-DNA insertions within the seventh exon and second intron, respectively (Fig. 5A). The insertion in *vtc4-3* occurs 1,037 nucleotides from the start site of translation, and *vtc4-4* contains two tandem T-DNA insertions at the end of exon 2 (Fig. 5A).

A lack of VTC4 expression was verified in the mutants by reverse transcription (RT)-PCR (Fig. 5B). Primers specific for an actin gene (*ACT8*) were used as a positive control (Fig. 5B). From this analysis, we conclude that

these mutants are suitable for examining the consequences of eliminating VTC4 expression. We examined the growth and development of *vtc4-2* and *vtc4-4* mutant lines grown in soil and present data for these mutants in

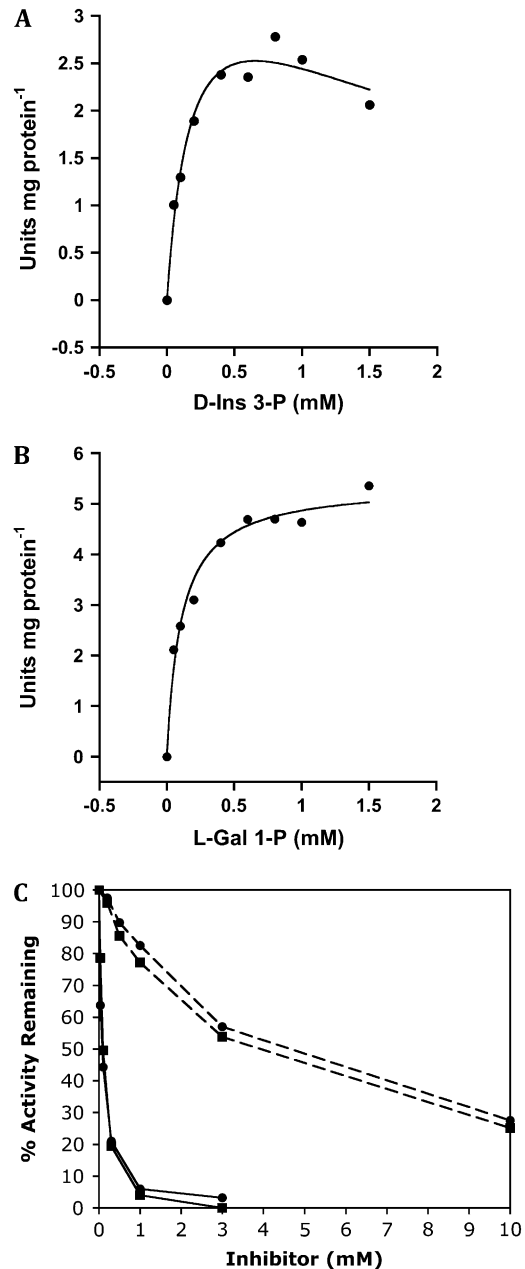


Figure 4. Kinetic analysis of VTC4 activity with D-Ins 3-P and L-Gal 1-P. Phosphatase activity (solid lines) was plotted versus varying concentrations of D-Ins 3-P (A) or L-Gal 1-P (B) in the standard reaction described in "Materials and Methods." Data were imported into Kaleidagraph (Synergy Software) and fit to a nonlinear curve with a substrate inhibition equation based on the Michaelis-Menten equation (A) or the Michaelis-Menten equation to calculate apparent K_m and V_{max} (B). C, Inhibition of VTC4 activity by either LiCl or $CaCl_2$. VTC4 activity was assayed with D-Ins 3-P (circles) or L-Gal 1-P (squares) in the presence of the indicated concentrations of $CaCl_2$ (solid lines) or LiCl (dashed lines).

Table II. Catalytic properties of VTC4

ND, Not determined; NR, not reported.

Sample	K_m		V_{max}	V_{max}/K_m Ratio	Reference
	μM	$\mu mol\ min^{-1}\ mg^{-1}\ protein$			
Arabidopsis (recombinant)					
D-Ins 3-P	191		4.0	0.029	This work
L-Gal 1-P	107		5.4	0.050	This work
Arabidopsis (partially pure)					
L-Gal 1-P	44 ^a				Laing et al. (2004)
Barley (recombinant)	9.7		NR		Fu et al. (2008)
Kiwifruit (partially pure)					
D-Ins 3-P	ND		NR		Laing et al. (2004)
L-Gal 1-P	41 ^b		NR		Laing et al. (2004)
Kiwifruit (recombinant)					
D-Ins 3-P	330		NR		Laing et al. (2004)
L-Gal 1-P	150		NR		Laing et al. (2004)
Lily					
D-Ins 3-P	78				Loewus and Loewus (1982)
Human					
D-Ins 3-P	75		36.8		McAllister et al. (1992)

^aAssays performed in 2 mM MgCl₂.^bAssays performed in 1.8 mM MgCl₂.

the following sections. Under standard laboratory conditions, *vtc4* mutants did not exhibit any major abnormalities in plant growth or development.

Metabolite Levels in *vtc4* Mutants

It has been suggested that VTC4 encodes an L-Gal 1-P phosphatase that functions exclusively during ascorbate synthesis (Laing et al., 2004). Our biochemical data support a bifunctional role of VTC4 in both L-Gal 1-P and D-Ins 3-P hydrolysis. Thus, it is of interest to examine the levels of both products (L-Gal and myo-inositol) in mutant plants to determine if in vivo, VTC4 is hydrolyzing both substrates. We extracted metabolites from control, *vtc4-4*, and *vtc4-2* leaves and subjected them to gas chromatography to quantify myo-inositol, D,L-Gal, and ascorbate (Fig. 6). The data indicate that a loss of function in VTC4 results in 22.4% and 34% decreases in mass myo-inositol contents in *vtc4-4* and *vtc4-2* mutants, respectively. In these same mutants, D,L-Gal levels increase 1.4- and 2.3-fold, respectively. In addition, ascorbate levels decline to 75% and 61% of wild-type leaf values. We conclude that a loss of function in the *imp* mutants reduces the myo-inositol product but increases the D,L-Gal product levels. This supports our in vitro data that indicate that VTC4 is capable of hydrolyzing InsPs and the general role of VTC4 in myo-inositol recycling and/or synthesis. Furthermore, we note that the decline in myo-inositol in these plants mirrors the decline in ascorbate also seen in these plants (Fig. 6).

Growth of *vtc4* Mutants

We have shown that a loss of function in the *VTC4* gene leads to a decrease in myo-inositol contents. Since

a reduction in myo-inositol synthesis could affect many processes such as myo-inositol phosphate signaling or ascorbate synthesis (Fig. 1), we tested whether known stress physiological pathways that utilize myo-inositol signaling (for review, see Xiong et al., 2002; Taji et al., 2006) were altered in *vtc4* mutants. We produced age-matched seed populations that had been harvested from plants grown at the same time. Control and mutant age-matched seeds were plated on Murashige

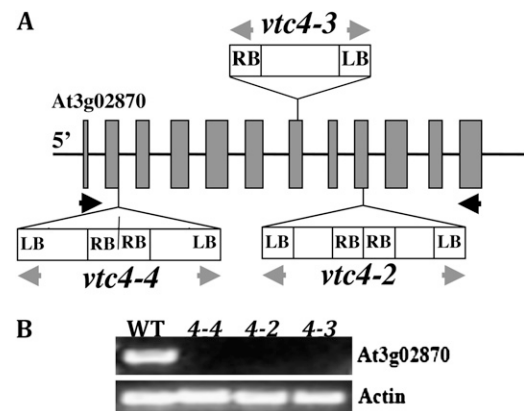


Figure 5. T-DNA insertions and loss of gene expression in mutant lines. A, Schematic of the T-DNA insertion sites in the *vtc4-2*, *vtc4-3*, and *vtc4-4* mutants. Exons in At3g02870 are shown as dark gray boxes; the gray arrows indicate primers used to amplify the right border (RB) and left border (LB) of the T-DNA; black arrows indicate the positions of gene-specific primers. B, Verification of the loss of VTC4 expression in mutant lines. Total RNA was isolated from leaves of 14-d soil-grown mutant and wild-type (WT) plants. RT-PCR was carried out with gene-specific primers for VTC4 and actin. Primer sequences can be found in "Materials and Methods."

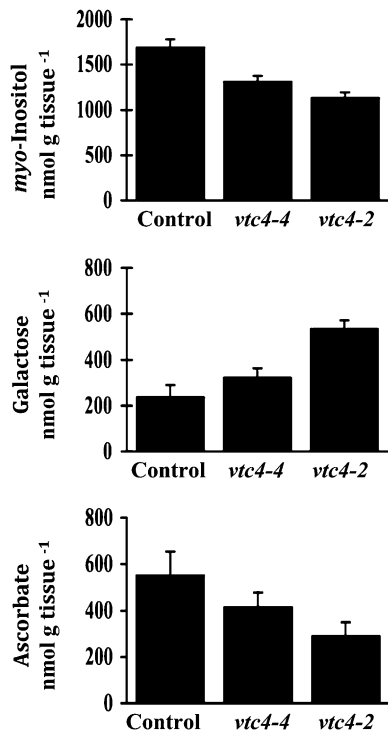
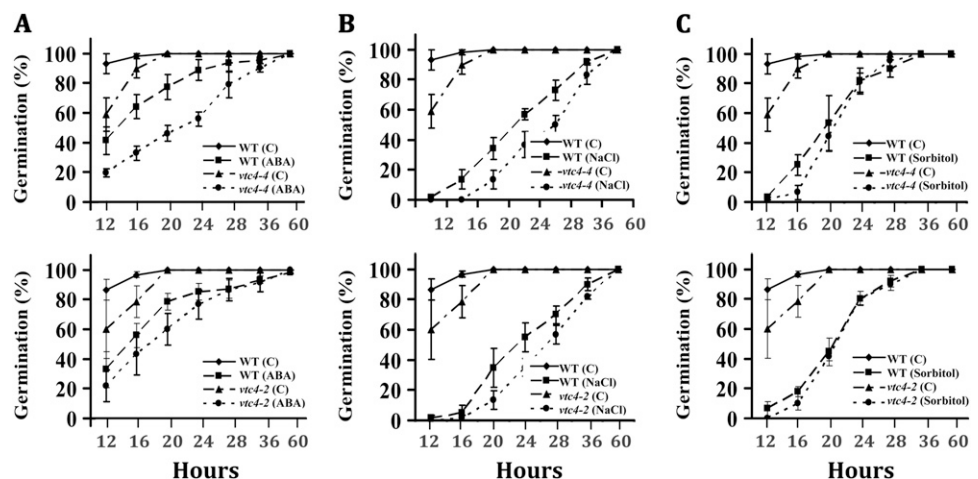


Figure 6. Metabolite levels in control and *vtc4* mutant plants. Leaf tissue from 14-d soil-grown plants was frozen in liquid nitrogen, ground, extracted, derivatized, and analyzed by gas chromatography as described in “Materials and Methods.”

and Skoog (MS) medium and stratified for 3 d at 4°C in the dark. To determine if a loss of function in *VTC4* alters abscisic acid (ABA), NaCl, or osmotic sensitivity during germination, we germinated mutants in the presence of 1.25 μM ABA, 150 mM NaCl, or 300 mM sorbitol and measured the impact on germination over 60 h. Our results (Fig. 7) indicate that *vtc4* mutants germinate slower under control conditions and are also slightly hypersensitive to ABA and NaCl during seed germination, as seen by the reduction in seed

Figure 7. Germination response of mutants to ABA, NaCl, and sorbitol stress. Wild-type CS60000 (WT; solid lines), *vtc4-4* and *vtc4-2* (dashed lines) seeds were plated on 0.5 \times MS agar medium with no additions or with 1.25 μM ABA (A), 150 mM NaCl (B), or 300 mM sorbitol (C). Seeds were stratified at 4°C for 3 d and then placed at 23°C under continuous light for the indicated times (hours), and germination was scored. Values represent means \pm SE of three replicates from one of three independent experiments.



germination over time. In contrast, *vtc4* mutant seeds do not differ from wild-type seeds in their response to sorbitol, an osmotic stress (Fig. 7).

Myoinositol phosphate signaling is also involved in the response to cold (Xiong et al., 2001; for review, see Zhu et al., 2007), so we tested whether *vtc4* mutants had alterations in cold responses. Seeds from wild-type and mutant plants were plated on MS medium and stratified for 3 d at 4°C. After stratification, plates were placed at 23°C (room temperature) or 4°C in the light, and germination was measured over a period of 14 d. No differences in germination rate were noted at 23°C; however, we found that *vtc4* mutants lag in germination when placed at 4°C as compared with wild-type seeds (Fig. 8A). To examine whether growth is also affected by the loss of function in the *VTC4* gene, we germinated seeds at 23°C on vertical plates and allowed root growth to proceed for 2 d before transfer to 4°C. New root growth of mutants was significantly decreased as compared with wild-type seedlings (Fig. 8B). Together, these experiments indicate that *vtc4* mutants have slower germination and root growth in the cold, which is indicative of cold sensitivity. Along with the alterations in ABA and salt sensitivity, these data indicate an overall increase in sensitivity to abiotic stresses known to involve myoinositol signaling and may be related to the decrease in myoinositol contents found in these plants.

Examination of IMPL Enzymes

We have shown that a loss of function in *VTC4* can impact myoinositol and ascorbate levels. However, *vtc4* mutants retain 66% to 78% of wild-type myoinositol contents, suggesting that other redundant enzymes function during myoinositol synthesis. The two IMPL proteins are good candidates for such enzymes, as they are the two most closely related proteins in the Arabidopsis genome as determined by a BLAST search. IMPL1 (At1g31190) and IMPL2 (At4g39120) contain the conserved inositol P domain found in all

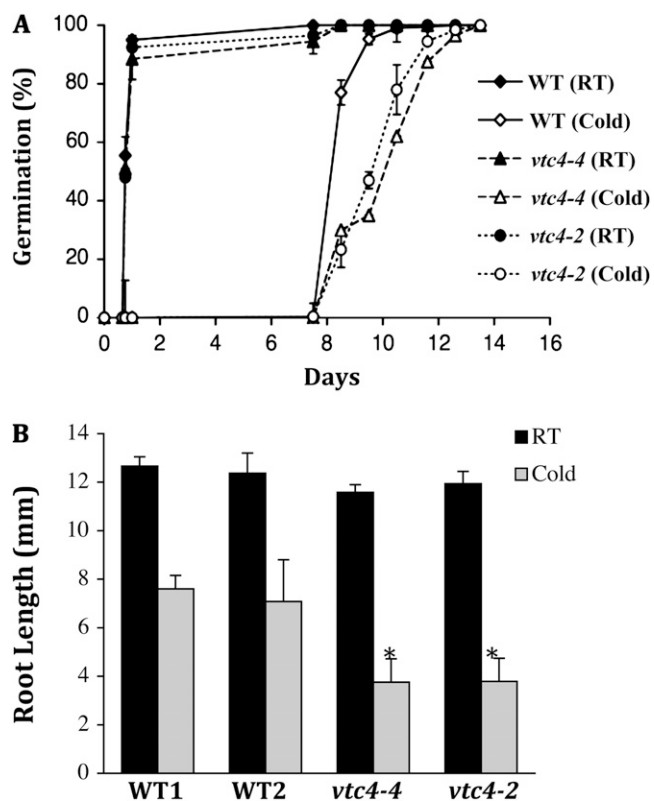


Figure 8. Cold sensitivity of *vtc4* mutants. Wild-type CS60000 (WT; solid lines) and *vtc4* mutant (dashed lines) seeds were plated on 0.5× MS agar medium, stratified at 4°C for 3 d, and then placed at 23°C (room temperature [RT]) or 4°C (Cold) under continuous light for the indicated times. Germination (A) and change in root length (B) were scored. Values represent means ± SE of three replicates ($n = 50$). In B, data from day 25 after transfer to the cold are presented. * $P < 0.05$ compared with the corresponding wild-type sample.

characterized IMPs and have sequence homology to the so-called bacterial IMPase domain.

To determine whether these are expressed genes, we analyzed the microarray data available in the Genvestigator database (Zimmermann et al., 2004) and found that both IMPL1 and IMPL2 are expressed in most Arabidopsis tissues (Supplemental Fig. S1A). To determine the phylogenetic relationship between the IMPL proteins and the plant and animal IMPs, we used ClustalW and PAUP4.0b to produce an unrooted, bootstrapped phylogenetic tree with 34 microbial, plant, and animal proteins containing the conserved inositol P domain (Supplemental Fig. S1B). As reported by others, we found that VTC4 and IMPL proteins are more related to other proteins than to one another, as seen by their presence on distinct branches of the tree (Supplemental Fig. S1B). VTC4 (At3g02870) is most related to the other plant IMPs and is found on a main branch containing the eukaryotic IMPs from animals, slime mold, and yeast. Many of these enzymes have been characterized biochemically. In contrast, IMPL1 (At1g31190) and IMPL2 (At4g39120) are

contained within the prokaryotic portion of the tree, indicating a closer relationship with the prokaryotic IMPs or SuhB proteins (Matsuhisa et al., 1995; Chen and Roberts, 2000). Specifically, IMPL1 is found on a branch with an uncharacterized *Aquifex aeolicus* protein, while IMPL2 is grouped with an uncharacterized *Halobacterium* protein.

To determine if IMPL1 and IMPL2 proteins are capable of hydrolyzing InsPs, truncated versions of IMPL1 and IMPL2 genes were expressed as glutathione S-transferase fusion proteins. The resulting recombinant proteins were purified using glutathione-Sephadex and were greater than 95% pure as observed by 12% SDS-PAGE (Fig. 2). As was noted for recombinant VTC4, both IMPL1 and IMPL2 migrated slightly slower than expected given their predicted molecular masses of 55.5 and 55.4 kD, respectively (Fig. 2). With three independent sets of purifications and phosphatase assays containing 50 mM Tris-Cl, pH 7.5, 0.4 mM substrate, 1 mM MgCl₂, and 0.5 μg of enzyme, IMPL1 catalyzed the reaction with D-Ins 1-P at 0.528 ± 0.105 units min⁻¹, with D-Ins 3-P at 0.0551 ± 0.0275 units min⁻¹, and with L-Gal 1-P at 0.0131 ± 0.0175 units min⁻¹. IMPL2 catalyzed the reaction with D-Ins 1-P at 0.361 ± 0.081 units min⁻¹, with D-Ins 3-P at 0.408 ± 0.001 units min⁻¹, and with L-Gal 1-P at 0.257 ± 0.004 units min⁻¹. These reactions show that IMPL2 may be more similar to VTC4 in its substrate preference, using the three substrates within a 2-fold difference in activity range. IMPL1, however, may be a D-Ins 1-P-specific enzyme, as it is 10-fold less active with D-Ins 3-P and 40-fold less active with L-Gal 1-P. We conclude that both IMPLs are capable of hydrolyzing InsP and could provide redundancy with respect to IMP function within the plant cell. Furthermore, IMPL2 is also a potential candidate for a plant L-Gal 1-phosphatase.

DISCUSSION

Since IMP traditionally occupies an integral role in both a metabolic synthesis pathway (the Loewus pathway) and in second messenger myoinositol (1,4,5)P₃ recycling, it provides a critical link between specific metabolic and signaling events in plant cells. Thus, to resolve whether the previously identified VTC4 really functions in myoinositol pathways or functions mainly as an L-Gal 1-phosphatase used for ascorbate synthesis is an important issue. Our kinetic characterization of the Arabidopsis VTC4 protein, along with data on loss-of-function mutants, indicates that VTC4 is bifunctional and can act to hydrolyze D-Ins 3-P, D-Ins 1-P, and L-Gal 1-P in vitro. In addition, *vtc4* mutant plants contain reduced myoinositol levels, indicating that a loss of VTC4 function impacts the supply of myoinositol in the plant cell.

Biochemical Evidence for IMP Bifunctionality

Previous reports of purified or recombinant kiwifruit VTC4 and partially purified AtVTC4 enzymes

indicated a large substrate preference for L-Gal 1-P, with a 12-fold difference in hydrolysis of L-Gal 1-P as compared with D-Ins 3-P by the Arabidopsis enzyme (Laing et al., 2004). In contrast, we showed that our purified, recombinant AtVTC4 protein had pH and MgCl₂ optima similar to previous studies and hydrolyzed L-Gal 1-P only 1.6- to 2.4-fold better than either D-Ins 3-P or D-Ins 1-P (Table I). There are two experimental differences that might account for this disparity. First, we are using different buffer conditions (Tris-HCl in our studies versus BisTris propane buffer used previously) and our MgCl₂ optimum was slightly higher (4 mM as compared with 1.8–2 mM). Therefore, it is possible that D-Ins 3-P and D-Ins1-P hydrolysis is more robust under our conditions.

We also report here the catalytic constants of VTC4 for InsP and L-Gal 1-P, which can be informative about substrate preference. The K_m values for L-Gal 1-P (107 μ M) and D-Ins 3-P (191 μ M) do not differ by more than 2-fold and are in good agreement with previously reported values (Table II; Loewus and Loewus, 1982; McAllister et al., 1992; Laing et al., 2004). As well, the V_{max}/K_m ratios between these two substrates show a 1.7-fold difference. This indicates, once again, a small difference in the substrate preference of VTC4 with respect to L-Gal 1-P and D-Ins 3-P. Together with the substrate comparisons (Table I), these data support a bifunctional role for the plant IMP.

Genetic Evidence for IMP Bifunctionality

To determine if a loss of IMP function impacts myoinositol synthesis, we isolated two independent T-DNA mutants defective in VTC4 gene expression (*vtc4-4* and *vtc4-2*) and found reductions in both ascorbate and myoinositol levels in these mutants (Fig. 6). This indicates that loss of VTC4 impacts myoinositol as well as ascorbate synthesis. The reduction in ascorbate is similar to that noted previously for the *vtc4-1* mutant, which was identified in a screen for ascorbate-deficient mutants. These authors found that *vtc4-1* mutants contained 42% of wild-type ascorbate levels along with reduced L-Gal 1-phosphatase activity (Conklin et al., 2006), which is in good agreement with the reduction we report here (Fig. 6). By measuring [³H]Man incorporation in *vtc4-1* mutants, these authors found that the *vtc4-1* mutation also disturbs L-Gal metabolism. They found an approximate doubling (from 11% to 24%) of label in L-Gal incorporated into polysaccharides and speculate that the VTC4 loss of function stimulates an increase in GDP-L-Gal incorporation into polysaccharides (Conklin et al., 2006). We also found evidence for a disturbance in Gal metabolism in *vtc4* mutants, namely an increase in free mass Gal levels (Fig. 6). Under the conditions we used for gas chromatography analyses, we cannot separate D-Gal and L-Gal; thus, we can only draw a limited conclusion. However, the trend of an increase in L-Gal in polysaccharides of *vtc4-1* mutants (Conklin et al., 2006) and in the soluble fraction of our *vtc4* mutants is

similar. Thus, one possibility to explain these results is that L-Gal 1-P accumulates in *vtc4* mutants and breaks down during derivatization for gas chromatography.

To determine if loss of VTC4 function alters physiology, we examined *vtc4* mutants for germination and root growth responses to ABA, salt, osmotic, and cold stress. We found small alterations in *vtc4* mutant responses to ABA, salt, and cold but no differences in response to osmotic stress (Figs. 7 and 8). These alterations could result from the small decrease in myoinositol contents, which could affect either myoinositol signaling or the production of downstream metabolites such as pinitol, a myoinositol derivative involved in osmoprotection (for review, see Taji et al., 2006). Alternatively, since ascorbate is important for detoxifying ROS generated from stress (for review, see Noctor, 2006), alterations in *vtc4* mutants may result from their lowered ascorbate levels.

Redundancy in L-Gal 1-P and InsP Phosphatases

Given that *vtc4* mutants still contain appreciable levels of myoinositol and ascorbate, it was important to determine whether other enzymes could function in myoinositol and/or ascorbate synthesis. IMPL1 and IMPL2 are the closest plant protein relatives of IMP, and phylogenetic analysis indicated their close relationship to the prokaryotic IMPs as well (Supplemental Fig. S1). Analysis of IMPL1 and IMPL2 activity with L-Gal 1-P, D-Ins3-P, and D-Ins 1-P identifies IMPL2 as another potential L-Gal 1-phosphatase from plants. In addition, both IMPL1 and IMPL2 provide potential redundancy in de novo myoinositol synthesis from D-Ins 3-P, while IMPL2 may also function in second messenger recycling pathways utilizing D-Ins 1-P. To resolve these issues, we need to determine the catalytic constants for these enzymes with different substrates. The purified IMPL2, in our hands, is unstable and efforts are under way to address this. In addition, we have not been able to recover a viable, homozygous, loss-of-function mutant for either the *AtIMPL1* or *AtIMPL2* gene, so we cannot currently test whether loss of these genes affects myoinositol or ascorbate synthesis. It is important to note that other plants, such as rice (*Oryza sativa*) and grape (*Vitis vinifera*), contain IMPL genes; thus, their contribution to myoinositol and ascorbate pathways could be conserved in all plants.

The Impact of IMP on Ascorbate Synthesis

The bifunctionality of VTC4 and IMPLs has important implications for understanding the synthesis of ascorbate in plants. Close inspection of the ascorbate pathway reveals two potential routes for VTC4 and IMPLs to influence ascorbate synthesis: one through L-Gal 1-P hydrolysis within the Smirnoff-Wheeler pathway (Smirnoff and Wheeler, 2000; Smirnoff, 2001; Smirnoff et al., 2001) and the other through D-Ins 3-P hydrolysis (Fig. 1). Frank Loewus pioneered studies in the myoinositol oxidation pathway, which contributes

to D-GlcUA synthesis via myo-inositol oxidation to ascorbate synthesis pathways in animals (for review, see Loewus, 2006). However, studies on strawberry (*Fragaria* species) and parsley (*Petroselinum crispum*) indicated that radiolabeled myo-inositol did not undergo conversion into ascorbate (Loewus et al., 1962; Loewus, 1963). Data from ectopic expression of the myo-inositol oxygenase gene in *Arabidopsis* indicates that this manipulation results in a 2- to 3-fold increase in ascorbate in transgenic plants, suggesting that myo-inositol can act as a precursor for ascorbate synthesis in a specific gain-of-function context (Lorence et al., 2004). In addition, InsP₆ hydrolysis by the phytase-like enzyme (AtPAP15) may also contribute myo-inositol for ascorbate synthesis, as shown by recent analyses of AtPAP15 mutants (Zhang et al., 2008). The impact of myo-inositol on ascorbate synthesis, however, is not sufficient to mitigate loss of the Smirnov-Wheeler pathway, as seen by recent work on GDP-L-Gal phosphorylase (Dowdle et al., 2007; Laing et al., 2007; Linster et al., 2007) double mutants (Dowdle et al., 2007). Double mutants in the two GDP-L-Gal genes are defective in postgermination seedling growth and can be rescued by exogenous ascorbate application (Dowdle et al., 2007).

From our work presented here, we show that *vtc4* mutants have reduced myo-inositol and ascorbate levels, which may indicate dual action of VTC4 on both L-Gal 1-P and D-Ins 3-P. While it is intriguing to note that VTC4 could theoretically act with two different substrates (L-Gal 1-P and D-Ins 3-P) to affect ascorbate synthesis, rigorous radiotracer experiments are needed to determine whether the reduction in myo-inositol contents directly affects ascorbate synthesis.

MATERIALS AND METHODS

Expression of Recombinant Protein

The *VTC4* gene (At3g02870) was amplified by PCR with primers 5'-ATGGCGGACAATGATTCTCT-3' (forward) and 5'-TCATGCCCTGTAAGCCGCA-3' (reverse). The template was generated by RT (Omniscript RT kit) of RNA extracted from wild-type plants using the RNeasy Plant Mini kit (both kits from Qiagen) according to the manufacturer's instructions. The resulting PCR product was cloned into plasmid pCART7/NT-TOPO using the pCR T7 TOPO TA Expression kit (Invitrogen). The newly created plasmid pAtIMPH contains the amplified At3g02870 gene (816 nucleotides) from the start ATG to the stop TGA along with upstream plasmid regions including a T7 promoter, ribosome binding site, ATG start site, 6xHIS region, Xpress epitope, and EK cleavage site. Because of the extra upstream plasmid sequences, the theoretical size of the protein is 36 amino acids longer than the 271 amino acids of IMP. pAtIMPH was used to transform TOP10F' *Escherichia coli* cells, and the gene sequence was verified. Similarly, plasmids containing the genes *IMPL1* (At1g31190) and *IMPL2* (At4g39120), designated pAtIMPL1H and pAtIMPL2H, respectively, were constructed. Genes were amplified by PCR with the same cDNA template and primers 5'-ATGGGAAGTCTCTAATAATT-3' (forward) and 5'-TTAAAGCTCTGTATGATAAT-3' (reverse) for *IMPL1* and 5'-ATGTTAGCTCAGTCGCACTT-3' (forward) and 5'-TCAATGCCACTCAAGTACT-3' (reverse) for *IMPL2*. The cloning and sequencing were similar to those performed for *VTC4*.

Plasmids containing the genes *IMPL1* (At1g31190) and *IMPL2* (At4g39120), truncated at the 5' end, were designated ptiMPL1AE and ptiMPL2AE. The truncation of *IMPL1* was accomplished by removing the coding region for the N-terminal 74 amino acids, then replacing the codon for the next amino acid

(G) with ATG. In a similar fashion, *IMPL2* was deleted in nucleotides coding for 76 amino acids, and the E codon was replaced with ATG. The genes were amplified by PCR from pAtIMPL1H and pAtIMPL2H plasmid templates and primer pairs 5'-ATAggattcATGGCTAAAACCACCGGAAC-3' (forward)/5'-CGCgaattcTTAAAGCTCTGTATGATAATC-3' (reverse) and 5'-ATAggattcATGCTTAGCGACTGAGCTG-3' (forward)/5'-GGCgaattcTCAATGCCACTCAAGT-3' (reverse), respectively (lowercase letters indicate restriction sites). The products were restriction digested with *Bam*HI and *Eco*RI and ligated to similarly digested pGEX2T (GE Healthcare). The plasmids are designed to express the truncated *IMPL1* and *IMPL2* fused to a C-terminal glutathione S-transferase. The plasmid sequences were verified by sequencing.

Overexpression of *VTC4* from pAtIMPH was induced in pAtIMPH-transformed host strain pREP4 BL21(DE3)*. A 1.5-L culture with optical density at 600 nm of 0.6, grown in Luria-Bertani medium with 100 $\mu\text{g mL}^{-1}$ ampicillin and 50 $\mu\text{g mL}^{-1}$ kanamycin, was induced with 0.1 mM isopropyl- β -D-thiogalactopyranoside for 5.5 h at room temperature without shaking. Cells were harvested by centrifugation and frozen at -80°C . All subsequent steps were performed at 4°C . Cells were resuspended in 20 mL of buffer L (50 mM potassium phosphate, 400 mM NaCl, 100 mM KCl, 10% glycerol, 0.1% Triton X-100, and 20 mM imidazole), pH 7.8, supplemented with 1 mg mL^{-1} lysozyme, 0.5 mM phenylmethanesulfonyl fluoride, and 1 mM myo-inositol. After incubation at 4°C for 45 min, cells were lysed through two passes with French pressure (12,000–16,000 psi) and centrifuged for 20 min. The cleared lysate was passed over a Qiagen nickel-nitrilotriacetic acid agarose column prepared using the manufacturer's instructions with 1 mM myo-inositol-supplemented buffer L. AtIMP was eluted from the column at pH 8 and room temperature with buffer L/1 mM myo-inositol/100 mM imidazole, after sequential washing with buffer L + 1 mM myo-inositol, buffer L/1 mM myo-inositol/56 mM imidazole, and buffer L/1 mM myo-inositol/70 mM imidazole. Fractions were collected and dialyzed extensively in 50 mM Tris-Cl, pH 7.5, 3 mM MgCl_2 , 250 mM KCl, 0.1 mM CaCl_2 (4°C). Purified IMP was frozen in aliquots at -80°C with 10% glycerol and 1 mM dithiothreitol. Protein purification and size were estimated by fractionation by 12% SDS-PAGE with respect to prestained markers (Bio-Rad).

Overexpression of *IMPL1* and *IMPL2* from ptiMPL1AE and ptiMPL2AE was induced in the host strain pREP4 BL21(DE3)* as described above. Cells were induced overnight at room temperature without shaking, harvested and frozen at -80°C , and then resuspended in 1 \times phosphate-buffered saline, pH 7.3, containing 1 mg mL^{-1} lysozyme. Cells were sonicated, and cleared lysate was incubated for 1 h with Pharmacia Glutathione Sephadex (GE Healthcare), washed with 1 \times phosphate-buffered saline with 0.1% Triton X-100, and then collected in a column. Protein was eluted with 10 mM glutathione in 50 mM Tris-Cl, pH 8.0, and assayed immediately.

Phosphatase Activity Assays

Phosphatase activity was determined by the inorganic phosphate quantitation assay (Lanzetta et al., 1979) with minor modifications. Standard conditions were 50 mM Tris-Cl, pH 7.5, 4 mM MgCl_2 , 0.4 mM substrate, and 1.5 μg of purified enzyme in a total reaction volume of 50 μL . Reactions were performed at room temperature (21°C – 25°C) for 30 s to 10 min, after which 800 μL of color reagent malachite green/ammonium molybdate solution was added to terminate the reaction. The A_{660} was determined by spectrophotometer. Control reactions without enzyme or without substrate were used to determine background phosphate levels, which were subtracted from experimental values. Enzyme-specific activity units are in μmol of phosphate. The assays to verify the pH optimum of reactions were performed in mixtures containing 4 mM MgCl_2 , while the assays to verify the optimum MgCl_2 concentration were performed in reaction mixtures of pH 7.5. Protein concentrations were determined as described by Bradford (1976) with bovine serum albumin as the standard.

Data from kinetic experiments were analyzed with Kaleidagraph software (version 4.0 Mac; Synergy Software). For Ins-1-P, data were fit to a nonlinear curve with a substrate inhibition equation based on the Michaelis-Menten equation. For L-Gal 1-P, data were fit to the Michaelis-Menten equation $v = k_{cat}[S]/(K_m + [S])$.

Mutant Isolation

Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia plants were maintained in a 1:1 mixture of Pro Mix (BX) and Pro Mix (PGX) in a growth room

set at 22°C/24°C night/day temperature. Visible radiation (100–320 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for 16 h) was provided by either a mixture of fluorescent/metal halide/high-pressure sodium lamps or fluorescent lamps only. Potential *vtc4* (At3g02870) mutants were identified from the Salk T-DNA lines (Alonso et al., 2003) through the analysis of the SiGnAL database (<http://www.signal.salk.edu/cgi-bin/tdnaexpress>). Seeds for *vtc4-2* (SAIL_105_D11), *vtc4-3* (SAIL_843_G10), and *vtc4-4* (SALK_077222) were obtained from the Ohio State University Arabidopsis Biological Resource Center. Corresponding wild-type plants CS60000 and CS908 were grown and maintained under similar conditions. Genomic DNA was isolated from the leaves of soil-grown plants. DNA from segregating plants was screened with PCR utilizing the SALK and SAIL left border primers 5'-GCCGTGGACCGCTTGCTGCAACT-3' and 5'-TTCATAACCAATCTCGATACAC-3', respectively, and the AtVTC4 forward and reverse gene-specific primers 5'-ATGGCGGACAATGG-TAAAGTC-3' and 5'-TCATGCCCTGTAAAGCCGCA-3', respectively, with annealing at 55°C. In the case of *vtc4-2*, a second left border forward primer PCR product was also apparent. The resulting PCR fragments were sequenced and compared with the genomic sequence for each gene to map the T-DNA insertions.

RT-PCR

Total RNA was extracted from leaf tissue of 35-d soil-grown wild-type and *vtc4* mutant plants using the RNeasy Plant Mini kit (Qiagen). One microgram of total RNA was reverse transcribed using the Qiagen Omniscript RT kit according to the manufacturer's instructions. Approximately one-tenth of the resulting mRNA eluate was used as template in each PCR, which was prepared in a 25- μL mixture. VTC4 was amplified at 55°C with the AtVTC4 reverse primer listed above and an internal primer, 5'-TGCAGCAG-GAATTGTATCG-3'. Actin amplification has been described (Berdy et al., 2001).

Seedling Growth and Seed Germination Assays

Age-matched seeds used for assays were harvested from plants grown in parallel on the same shelf in a growth room, and seeds were harvested on the same day and ripened for 42 d at room temperature. Seeds were surface sterilized and plated on 0.5 \times MS salts solution (pH 5.8) containing 0.8% agarose. Seeds were stratified on plates at 4°C for 3 d and germinated at 23°C in the light (100 μE). Germination was scored as positive when the radicle protruded through the seed coat. For ABA sensitivity experiments, ABA (Sigma) was dissolved in 100% ethanol and added to medium at a final concentration of 0.25, 0.5, 1.25, or 2 μM ABA. Hormone treatment experiments were repeated three times. For salt and osmotic sensitivity experiments, medium was supplemented with 150 mM NaCl or 300 mM sorbitol. Treatment experiments were repeated three times. For cold sensitivity tests, seeds were sown in a straight line on MS agar plates, and after stratification, plates were grown vertically for 3 d at 22°C under the light at 100 $\mu\text{E m}^{-2} \text{s}^{-1}$. Root lengths were marked, and the plates were transferred to 4°C for 25 d. Measurements of change in root length were made every 2 d.

Gas Chromatography Analysis

Frozen seedlings and tissues were ground into a powder, and 1 mL of ethanol was mixed with the powder. Two milligrams of D-chiroinositol was added to the mixture as an internal standard. The mixture was incubated at 70°C for approximately 1.5 h. The insoluble portion was removed by centrifugation. The supernatant was dried in a speed-vacuum chamber (Savant) and reconstituted in 200 μL of water, filtered through a 0.2- μm Tuffryn syringe filter (Pall Gelman Laboratory), and dried again. Derivatization reagent (1:1 mixture of pyridine and *N,O*-bis(trimethylsilyl)trifluoroacetamide + 1% trimethylchlorosilane; Alltech) was freshly prepared. For sample derivatization, 250 μL of the derivatization reagent was added to the dried sample. The sample was sonicated and heated at 80°C for at least 15 min until all of the sample was in solution. The sample was transferred to an autosample vial, and 250 μL of hexane was added to the sample. The sample was then injected with a split of 10 mL min^{-1} and separated by gas chromatography (Perkin-Elmer Instruments) on a Rtx-5 fused capillary column (30 m \times 0.32 mm i.d.; Restek) with helium as the carrier gas, pressure-controlled flow set at 6.5 psi, and a linear velocity of 1 mL min^{-1} . The injection port was set at 225°C, the oven was set on a gradient from 75°C to 274°C at 6.5°C min^{-1} , and the flame ionization detector was set at 280°C. The myoinositol, L-ascorbate, and Gal

levels were calculated based on standard curves for each of the compounds and recovery of the internal standard. Gas chromatography-mass spectrometry was used to confirm the identity of myoinositol, ascorbate, and Gal peaks.

Phylogenetic Analyses

ClustalW was used to align amino acid sequences of representative proteins containing the inositol P domain. PAUP 4.0b was used to create an unrooted bootstrapped phylogenetic tree using maximum parsimony. Percentage confidence levels for branches (derived from 500 bootstrap trees) were determined.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers NP_001118558, NP_195623, and NP_564376.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Expression of IMPL proteins and phylogenetic analysis.

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