

## Chapter 2

# Inositol and Plant Cell Wall Polysaccharide Biogenesis

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### 1. INTRODUCTION

To the best of our knowledge, cyclization of D-glucose-6-phosphate (Glc-6-P) to 1L-*myo*-inositol-1-phosphate (MI-1-P) by *myo*-inositol-1-P synthase (MIPS, EC 5.5.1.4), followed by dephosphorylation of the latter by a specific MI monophosphatase (MIPase, EC 3.1.1.25), constitutes the sole *de novo* route to free MI in plants. Other sources of free MI involve salvage mechanisms on metabolic products bearing an intact MI structure. Examples include phosphoinositide biochemistry (Stevenson *et al.*, 2000) and hydrolysis of MI-containing polyphosphates or glycosides such as phytic acid and galactinol (Ercetin and Gillaspay, 2002; Hitz *et al.*, 2002; Loewus, 1973a,b, 2002; Loewus and Loewus, 1980; Loewus and Murthy, 2000; Morré *et al.*, 1990; Styer *et al.*, 2004).

Free MI undergoes phosphorylation by a  $Mg^{2+}$ -activated, ATP-dependent MI kinase (MIK, EC 2.7.1.64) to yield MI-1-P of the same stereoisomeric form produced by MIPS (English *et al.*, 1966; Loewus, M.W. *et al.*, 1982). For an introduction to current recommendations on rules for numbering atoms in *myo*-inositol see <http://www.chem.qmul.ac.uk/iupac/cyclitol/myo.html>.

Whether separate pools of MI-1-P are generated, one from *de novo* biosynthesis, another from the action of MIK on recycled free MI, needs to be explored. Evidence is accumulating to suggest the presence of several independent sites of MI-1-P formation within cells and tissues (Benaroya *et al.*, 2004; Hegeman *et al.*, 2001; Lackey *et al.*, 2002, 2003; Yoshida *et al.*, 1999). Multiple forms of MIPase are also found (Gillaspay *et al.*, 1995; Styer *et al.*, 2004). Quite possibly,

free MI from both sources intermingle once produced. Alternatively, dedicated metabolic processes limit such mixing.

Free MI is required for many biosynthetic processes in plants including formation of the raffinose series of oligosaccharides (Obendorf, 1997), biosynthesis of isomeric inositols and their *O*-methyl ethers (Miyazaki *et al.*, 2004), and membrane biogenesis (Collin *et al.*, 1999). Many of the inter-relationships that govern production and utilization of free MI have yet to be sorted out (Karner *et al.*, 2004). In addition to processes noted above wherein the inositol structure is conserved, at least one major catabolic process competes for free MI. MI oxygenase (MIOase, EC 1.13.99.1) was first discovered in kidney tissue (Charalampous and Lyras, 1957; Howard and Anderson, 1967). Here, the carbocyclic ring of MI is oxidized between carbon 1 and carbon 6 with incorporation of a single atom of O<sub>2</sub> exclusively into CO<sub>2</sub>H of the product, D-glucuronic acid (GlcUA). In animals, GlcUA is successively converted in subsequent steps to L-gulonate, 3-oxo-L-gulonate, L-xylulose, xylitol, D-xylulose, and D-xylulose-5-P, which then enters the pentose phosphate cycle.

Evidence for MIOase in plants has relied largely on experiments involving the comparative use of radiolabeled MI and D-glucose (Glc) as markers to follow their relative roles as precursors of UDP-GlcUA and its metabolic products (Loewus and Loewus, 1980). The recent report of a MIOase gene in chromosome 4 (*miox4*) of *Arabidopsis* and confirmation of its enzymatic activity as bacterially expressed recombinant protein provides important new evidence in this regard (Lorence *et al.*, 2004).

This review will examine experimental evidence for participation of MI in plant cell wall biogenesis in an attempt to consolidate a long-standing viewpoint (Loewus, 1973a) that oxidation of MI provides an alternate starting point to a pathway furnishing uronosyl and pentosyl residues for cell wall biogenesis.

## 2. HISTORICAL PERSPECTIVE

Serendipitous discovery of a MI oxidation pathway (MIOP) to uronosyl and pentosyl units of plant cell wall polysaccharides emerged from experiments on the biosynthesis of L-ascorbic acid (AsA) which sought to generate GlcUA from labeled MI *in situ* in plant tissues. Isherwood *et al.* (1954) had proposed a biosynthetic pathway (D-galactose → D-galacturonic acid → L-galactonic acid → L-galactono-1,4-lactone → L-ascorbic acid) in plants similar to one (D-glucose → D-glucuronic acid → L-gulonic acid → L-gulono-1,4-lactone → L-ascorbic acid) proposed earlier for ascorbic acid-synthesizing animals (summarized by Burns, 1967). Both schemes predicted an inversion of the six-carbon chain between the sugar precursor and the product, AsA. While the animal pathway did exhibit such an inversion (Loewus *et al.*, 1960), no inversion was observed when [1-<sup>14</sup>C]Glc or D-[1-<sup>14</sup>C]galactose ([1-<sup>14</sup>C]Gal) was supplied to detached strawberry fruit (Loewus, 1961). Subsequent studies on this tissue

with D-[1- $^{14}\text{C}$ ]- or D-[6- $^{14}\text{C}$ ]glucuronolactone resulted in AsA labeled predominately in carbon 6 or carbon 1, respectively, clearly an inversion of the carbon chain. In a similar experiment, D-[1- $^{14}\text{C}$ ]galacturonic acid-labeled strawberry fruit also produced AsA labeled predominately in carbon 6 (Loewus and Kelly, 1961). D-[1- $^{14}\text{C}$ ]Glucuronolactone-labeled berries also produced  $^{14}\text{C}$ -labeled uronosyl and pentosyl residues in cell wall polysaccharides (Finkle *et al.*, 1960). These novel studies provided first evidence of at least two separate pathways of AsA formation in higher plants, findings now established by identification and characterization of key enzymes involved in these pathways (Agius *et al.*, 2003; Lorence *et al.*, 2004; Smirnoff *et al.*, 2001, 2004).

To examine relative roles of hexose and uronic acid as AsA precursors, the possibility of generating labeled GlcUA from [2- $^3\text{H}$ ]- or [2- $^{14}\text{C}$ ]MI *in situ* in strawberry fruit was tested (Loewus, 1965; Loewus *et al.*, 1962). In [2- $^3\text{H}$ ] MI-labeled strawberry fruit, 40% of the  $^3\text{H}$  was recovered in free D-xylose (Xyl) and uronosyl and pentosyl residues of pectin. In [2- $^{14}\text{C}$ ] MI-labeled strawberry fruit, 33% of the  $^{14}\text{C}$  was recovered in these products. Only trace amounts of  $^3\text{H}$  or  $^{14}\text{C}$  appeared in AsA (Loewus *et al.*, 1962). D-Galacturonosyl, D-xylosyl, and L-arabinosyl residues of cell wall polysaccharides as well as free Xyl were degraded to establish the location of the radiolabel. In each instance, it was at carbon 5 (Loewus and Kelly, 1963).

To explore the potential of MI as a precursor of uronosyl and pentosyl residues in hemicellulose, germinating barley, a tissue rich in this cell wall polymer was used (Loewus, 1965). Two-day-old seedlings were labeled with [2- $^{14}\text{C}$ ] MI by placing the radioactive solution directly on root hairs. After 32 h, tissues were repeatedly extracted with 70% ethanol to remove soluble  $^{14}\text{C}$ , and then treated successively with pectinase, rumen bacterial extract, and dilute sulfuric acid. Washed residue was extracted with 3.5 N NaOH for 16 h at 25°. Insoluble residues were removed and the clear supernatant neutralized and freed of salts. A slight precipitate of hemicellulose A was removed and three volumes of ethanol added to the neutral solution to recover hemicellulose B. This fraction contained four times as much  $^{14}\text{C}$  per unit weight as the preceding hydrolytic steps. Hydrolysis of a portion of this hemicellulose B with 3 N HCl followed by chromatographic separation showed  $^{14}\text{C}$  confined to uronosyl, arabinosyl, and xylosyl residues. Virtually all  $^{14}\text{C}$  present in each of these three products was in carbon 5.

The similarity between products of MI metabolism and those of GlcUA metabolism supported the view that the first step in MI catabolism in plants was an oxidative cleavage to form GlcUA. It also provided an experimental means of demonstrating that Glc cyclized to MI with no conformation changes in the carbon chain (Fischer, 1945; Loewus, 1974). Additional experiments were undertaken to confirm this finding. Stem-fed detached parsley leaves were labeled with [1- $^{14}\text{C}$ ]Glc (Loewus, 1965; Loewus and Kelly, 1962). After a period of metabolism, labeled MI, sucrose, pectin, and AsA were recovered from leaf extracts. The glucose moiety of sucrose, galacturonosyl residues of pectin, and AsA each had about 80% of their  $^{14}\text{C}$  in carbon 1, the original position

of the supplied radiolabeled glucose. Partial redistribution of  $^{14}\text{C}$ , primarily between carbon 1 and carbon 6 of labeled constituents, is a process characteristic of hexose/triose phosphate metabolism (Krook *et al.*, 1998, 2000; Shibko and Edelman, 1957). The labeled MI was injected into ripening strawberry fruits where it was utilized for pectin biosynthesis (Loewus and Kelly, 1963). This labeled pectin was hydrolyzed to recover its labeled galacturonosyl and pentosyl residues, which were degraded to determine the location of  $^{14}\text{C}$  in the carbon chain. Again, about 80% of the  $^{14}\text{C}$  in the galacturonate was in carbon 1 and corresponded to carbon 6 of the injected MI. In other words, MI recovered from [ $^{14}\text{C}$ ]Glc-labeled parsley leaves had 80% of its  $^{14}\text{C}$  in the same position as the labeled Glc used for tagging the leaves.

Prior to the discovery that MI catabolism in plants produced uronosyl and pentosyl residues of cell wall polysaccharides such as pectin and hemicellulose, it was largely assumed that the sole pathway to these products arose from oxidation of UDP-Glc to UDP-GlcUA (Davies and Dickinson, 1972) and its subsequent metabolism (Feingold, 1982; Loewus and Dickinson, 1982). Once evidence for cyclization of Glc to MI in plants and animals was obtained (Eisenberg *et al.*, 1964; Loewus and Kelly, 1962) and the enzyme MIPS that catalyzed this process was isolated and characterized (Loewus and Loewus, 1971, 1973a,b; 1974; Sherman *et al.*, 1981), it was possible to construct an alternative pathway to these cell wall polysaccharides that bypassed UDP-Glc dehydrogenase (UDP-GlcDHase, EC 1.1.1.22). These two pathways, conveniently referred to as the MIOP and the sugar nucleotide oxidation pathway (SNOP), are shown in Figure 1. Absent from this figure is a proposed scheme linking oxidation of MI to AsA biosynthesis (Lorence *et al.*, 2004). Section 4.3 of this review provides an overview of this new development and its possible involvement in products of AsA catabolism, notably, oxalic acid, and tartaric acid (Bánhegyi and Loewus, 2004; DeBolt *et al.*, 2004).

From a practical viewpoint, radiolabeled MI is a useful tool for marking uronosyl and pentosyl residues of pectin, hemicellulose, and related plant cell wall polysaccharides. A wide variety of plant tissues have been studied with this procedure including cell and algal cultures, aquatic plants, germinating seeds, root tips, vascular and leaf tissue, floral parts and exudates, germinating pollen, ripening fruit, and seed development (Albersheim, 1962; Asamizu and Nishi, 1979; Harran and Dickinson, 1978; Imai and Terashima, 1991, 1992; Imai *et al.*, 1997, 1998, 1999; Knee, 1978; Kroh and Loewus, 1968; Kroh *et al.*, 1970a,b, 1971; Labarca and Loewus, 1970, 1972, 1973; Labarca *et al.*, 1973; Loewus, 1965; Loewus and Kelly, 1963; Loewus and Labarca, 1973; Loewus *et al.*, 1962, 1973; Maiti and Loewus, 1978a,b; Manthey and Dickinson, 1978; Mattoo and Lieberman, 1977; Roberts and Loewus, 1966, 1968, 1973; Roberts *et al.*, 1967a,b, 1968; Sasaki and Loewus, 1980, 1982; Sasaki and Taylor, 1984, 1986; Seitz *et al.*, 2000; Verma and Dougall, 1979; Wakabayashi *et al.*, 1989). Furthermore, synthesis of perdeuterated MI has provided a marker for stoichiometric evidence of the MIOP (Sasaki and Nagahashi, 1990; Sasaki *et al.*, 1989).



early stages of purification from plant extracts. It has high affinity for both 1D- and 1L-MI-1-P and a much lower affinity for MI-2-P (Loewus and Loewus, 1982). Recent studies on the effect of MI and  $\text{Li}^+$  on regulation of *LeIMP-1* and *LeIMP-2* genes in tomato suggest that these substances alter expression as measured by GUS staining (Styer *et al.*, 2004).

### 3.1.3 Step 3: MI oxygenase (MIOase, EC 1.13.99.1)

Of the five enzymatic steps in the MIOP in plants, four have been isolated and their properties reported. Plant MIOase remains a challenge although its counterpart in animal tissues has been actively investigated for over 37 years (Arner *et al.*, 2001; Howard and Anderson, 1967; Reddy *et al.*, 1981). Arner isolated and sequenced a cDNA clone encoding MIOase from pig kidney and expressed the rMIO protein in bacteria. Their enzyme, a 32.7-kDa protein, lacked significant sequence to other known proteins. Native pig MIOase appears to complex with GlcUA reductase to produce L-gulonate, the second intermediate leading to AsA in AsA-synthesizing animals.

The pathogenic yeast, *Cryptococcus neoformans*, synthesizes MI and catabolizes this cyclitol to GlcUA. These pathways regulate in opposite modes, repressing conditions for one are inducing conditions for the other (Molina *et al.*, 1999). More recently, a non-pathogenic species, *C. lactativorus*, which grows on MI as its sole energy source, provided the advantage that MIOase can be induced by MI. MIOase is absent in Glc-grown cells but is present in MI-grown cells. This organism was used to purify, characterize, and clone MIOase (Kanter *et al.*, 2003).

Recently, Kanter *et al.* (2005) discovered a gene family of MIOases in *Arabidopsis* that contribute to the pool of nucleotide sugars for synthesis of plant cell wall polysaccharides.

Molecular evidence that a functionally unassigned open reading frame in *Arabidopsis* does, in fact, encode a putative MIOase has just been reported (Lorence *et al.*, 2004). In this study, MIOase provides a possible entry point into AsA biosynthesis. Those concerned with MIOase and its role in the MIOP will now have the opportunity to extend these findings to breakdown products of AsA catabolism (Bánhegyi and Loewus, 2004) as well as to cell wall biogenesis.

2-*O,C*-Methylene-MI (MMO) is one of several MI antagonists that produce morphological modifications in *Schizosaccharomyces pombe*, a fission yeast with an absolute requirement for MI (Schopfer *et al.*, 1969). When this epoxide is injected into rats, MIOase is inactivated and necrotic lesions develop in the kidneys. Simultaneous administration of MI prevents enzyme inactivation and reduces cytotoxicity (Weinhold and Anderson, 1967). The epoxide also represses pollen germination, inhibits pollen tube elongation, and delays seed germination (Chen *et al.*, 1977; Maiti and Loewus, 1978a,b).

#### **3.1.4 Step 4: GlcUA 1-kinase (GlcUAKase, EC 2.7.1.43)**

This enzyme was first isolated from mung bean seedlings (Neufeld *et al.*, 1959). Ungerminated lily pollen (Dickinson, 1982) is an abundant source and the activity of GlcUAKase does not increase during germination (Dickinson *et al.*, 1973). The enzyme is competitively inhibited by its product,  $\alpha$ -D-glucuronate-1-P, and by UDP-GlcUA (Gillard and Dickinson, 1978; Leibowitz *et al.*, 1977). Since both inhibitors are intermediates in the MIOP, their effects may have regulatory significance in cell wall formation.

#### **3.1.5 Step 5: GlcUA-1-P uridylyltransferase (GlcUAUase, EC 2.7.7.44)**

UDP-GlcUA is a product common to two pathways (Figure 1, Steps 1–5 and Steps 6–8). In the MIOP, it is produced from GlcUA-1-P by GlcUAUase while in the SNOP it is produced from UDP-Glc by UDP-Glc dehydrogenase. It is not known if the products from these two pathways commix or if the pathways involved represent separate intracellular compartments. The plant enzyme was partially purified from barley seedlings and characterized by Roberts (1971). Subsequently, a modification in the assay for enzymatic activity provided enhanced detection (Dickinson *et al.*, 1977). A survey of GlcUAUase levels in a number of plant tissues suggests that this enzyme is present in amounts well in excess of that required to maintain requirements for cell wall biosynthesis and in this respect points to Steps 3 and 4 as potential rate-limiting steps (Dickinson *et al.*, 1977; Roberts and Cetorelli, 1973).

### **3.2 Sugar nucleotide oxidation pathway (SNOP, Steps 6–8)**

#### **3.2.1 Steps 6 and 7: phosphoglucomutase (PGMase, EC 5.4.2.2) and UTP:Glc-1-P uridylyltransferase (EC 2.7.7.9)**

Activation of Glc-6-P to form UDP-D-Glc (Steps 6 and 7) provides the glucosyl donor requirement for UDP-D-glucuronosyl units and related nucleotides in polysaccharide biosynthesis via the SNOP (Feingold, 1982; Feingold and Avigad, 1980). Both cytosolic and plastidic isoforms of PGMase occur in plants (Davies *et al.*, 2003; Periappuram *et al.*, 2000). A recent histochemical analysis of PGMase in *Arabidopsis* found several organ-specific quantitative trait loci (Sergeeva *et al.*, 2003).

UDP-Glc is the principal sugar nucleotide and UTP:Glc 1-P uridylyltransferase is the major sugar nucleotide transferase in plant tissues (Feingold, 1982). Apart from its role in the SNOP, the transferase functions as glycosyl donor to sucrose, starch, callose, and cellulose (Hopper and Dickinson, 1972; Schlüpmann *et al.*, 1994; Tenhaken and Thulke, 1996). UDP-Glc inhibits this

enzyme, contributing to control of its own production and thus regulating flow of hexosyl units toward formation of major cellular glycosides as well as toward cell wall polysaccharides.

### **3.2.2 Step 8: UDP-D-glucose dehydrogenase (EC 1.1.1.22)**

UDP-D-Glucuronate (UDP-GlcUA), product of Step 8, is also the final step (Step 5) of the MIOP. As such, the potential impact of this biosynthetic juncture on cell wall growth and development cannot be ignored. Historically, efforts to probe relative contributions of the MIOP and SNOP to UDP-GlcUA weighed heavily on the side of the SNOP (Davies and Dickinson, 1972; Feingold, 1982; Gibeaut *et al.*, 2001; Hinterberg *et al.*, 2002; Johansson *et al.*, 2002; Loewus and Dickinson, 1982; Robertson *et al.*, 1995; Stewart and Copeland, 1998; Tenhaken and Thulke, 1996) but this is likely to change as more chemical and molecular data *a propos* the MIOP emerge (Kanter *et al.*, 2005; Kärkönen, 2005; Kärkönen/*et al.*, 2005; Loewus and Loewus, 1980; Morr   *et al.*, 1990; Seitz *et al.*, 2000).

It is worth noting here that “. . . UDP-Glc dehydrogenase is a good candidate for a control point in the metabolic pathway of cell wall synthesis not only because it is in low concentration relative to other enzymes in the pathway, operates far from equilibrium, and because so much of the cell wall carbohydrate is acted on by this enzyme . . .” (Gibeaut *et al.*, 2001) but also because it is strongly inhibited by its product, as well as a subsequent product, UDP-Xyl, leading to speculation that tissue-specificity determines functional contributions of the MIOP and SNOP (Davies and Dickinson, 1972; Harper and Bar-Peled, 2002; Hinterberg *et al.*, 2002).

### **3.3 Relative contributions of the MIOP and SNOP to plant cell wall biogenesis**

Ambivalence regarding a functional role for the MIOP in plant cell wall polysaccharide biosynthesis still lingers in current literature (Doblin *et al.*, 2003; Gibeaut, 2000; Mellerowicz *et al.*, 2001; Reiter, 2002; Reiter and Vanzin, 2001; Ridley *et al.*, 2001), in large part due to scarce attention given to molecular aspects of this alternative pathway (Kanter *et al.*, 2005; Seitz *et al.*, 2000). This despite a seemingly ubiquitous occurrence of the MIOP in plants as emphasized by a growing list of plant tissues that absorb labeled MI and metabolize it to cell wall uronides and pentoses (see Section 2). A survey of earlier literature is summarized in following sections.

#### **3.3.1 Pectin synthesis in oat seedlings and ripening apples**

Experiments with oat seedlings concluded that MI and Glc pass through a common precursor of pectin and that the rate of pectin synthesis was limited by



a reaction subsequent to that intermediate. Moreover, the rate of incorporation of MI into GalUA residues of pectin was equivalent to that of Glc (Albersheim, 1962). Studies involving pectin synthesis in cortical slices from ripening apples incubated in sucrose media containing  $[2\text{-}^3\text{H}]\text{MI}$  or  $[^{14}\text{C}]\text{methyl-L-methionine}$  also gave comparable rates (Knee, 1978).

### **3.3.2 Influence of MI on redistribution of $^{14}\text{C}$ from $[1\text{-}^{14}\text{C}]\text{Glc}$ in parsley leaf pectin and starch**

When detached parsley leaves were labeled with  $[1\text{-}^{14}\text{C}]\text{Glc}$ , then transferred either to water or to 1% MI and allowed to metabolize for 42 h, both MI and pectin-derived galacturonosyl residues from such leaves retained about 80% of the  $^{14}\text{C}$  in the original position while remaining label appeared in the other terminal position. In contrast to this, the distribution pattern of  $^{14}\text{C}$  in sucrose-derived Glc from these same leaves was greatly influenced by the presence of excess MI which caused redistribution of 40% of the  $^{14}\text{C}$  from carbon 1 into carbon 6 (Loewus, 1965). These results may be interpreted as evidence favoring operation of the MIOP where, in the presence of excess MI, more labeled Glc is available to equilibrate with triose phosphate (Krook *et al.*, 1998, 2000). The fact that this excessive redistribution of  $^{14}\text{C}$  in the 1% MI enriched experiment, occurred only in sucrose-derived Glc, a product of UDP-Glc, but not in galacturonosyl residues of pectin, a product of UDP-GlcUA, is significant (Roberts *et al.*, 1968). In a comparable study involving  $[6\text{-}^{14}\text{C}]\text{Glc}$ -labeled root tips from three-day-old *Zea mays* seedlings, raising the internal concentration of MI did not greatly influence the pattern of Glc uptake and  $\text{CO}_2$  release yet greatly reduced the flow of label into galacturonosyl units of pectin as well as glucuronosyl units of hemicellulose. As expected, pentosyl units of pectin and hemicellulose which arose from decarboxylation of UDP-GlcUA at Step 9 (Figure 1) were essentially unlabeled (Roberts and Loewus, 1973).

### **3.3.3 Hydrogen isotope effect in MIPS biosynthesis**

In another study designed to probe the functionality of the MIOP, use was made of a hydrogen isotope effect at carbon 5 of Glc-6-P by MIPS (Loewus, M.W 1977). MIPS prepared from sycamore maple cell- (Loewus and Loewus, 1971) or rice cell-cultures (Funkhouser and Loewus, 1975; Loewus *et al.*, 1978) converted  $[5\text{-}^3\text{H}]\text{Glc-6-P}$  to  $[2\text{-}^3\text{H}]\text{MI}$  at rates ranging from 0.2 to 0.5 that of unlabeled substrate, an isotope effect indicating involvement of carbon 5 of Glc-6-P in MI biosynthesis (Loewus *et al.*, 1978). Comparison of  $^3\text{H}/^{14}\text{C}$  ratios in glucosyl and galacturonosyl residues of starch and pectin, respectively, from germinating lily pollen digests, provided meaningful evidence of a functional role for the MIOP (Loewus, M.W. and Loewus, 1980).

### **3.3.4 Comparative study of MMO inhibition of MIOase in germinating lily pollen and wheat**

As mentioned earlier (Section 3.1.3), MMO, an inhibitor of MIOase, repressed lily pollen germination and tube elongation. When excess MI was included in the germination medium, MMO effects were partially blocked or, if MI was supplied subsequent to MMO inhibition, reversed (Chen and Loewus, 1977; Chen *et al.*, 1977). MMO did not inhibit MIPS or UDP-Glc dehydrogenase. When [2-<sup>3</sup>H]MI was included in the growth medium of lily pollen, <sup>3</sup>H rapidly incorporated into uronosyl and pentosyl units of tube wall polysaccharides, primarily pectic components. MMO blocked this process. Uptake of Glc by germinating lily pollen was not altered in the presence of MMO. Pollen grains germinated in pentaerythritol-balanced, sucrose-free media (Dickinson, 1978) containing [1-<sup>14</sup>C]Glc produced labeled pollen tubes with <sup>14</sup>C-labeled glucosyl, galactosyl, uronosyl, and pentosyl units in their tube wall polysaccharides. When MMO was present in the media, incorporation of <sup>14</sup>C into glucosyl and galactosyl units was unaffected but incorporation of <sup>14</sup>C into uronosyl and pentosyl units was greatly repressed (Loewus *et al.*, 1973). These results provided further evidence in support of a functional role for the MIOP in pollen tube wall biogenesis.

### **3.3.5 Comparative labeling of germinating lily pollen with [2-<sup>3</sup>H]MI or [1-<sup>14</sup>C]Glc**

Further evidence for the MIOP was obtained by germinating lily pollen in pentaerythritol media, a non-metabolized poly-hydroxylated osmoticum (Dickinson, 1978). Pollen tubes, grown for 3 h to deplete endogenous levels of MI and Glc, were resuspended in growth media containing 5.6–28 mM Glc with a trace of [2-<sup>3</sup>H]MI or in media containing 5.6–28 mM MI with a trace of [1-<sup>14</sup>C]Glc. After 3 h in labeled media, tubes were ground in 70% ethanol and tube walls recovered. Walls were treated successively with amyloglucosidase and pectinase to recover starch-derived Glc and pectin-derived uronosyl and pentosyl units by chromatography. In the presence of high levels of Glc, the [2-<sup>3</sup>H]MI-labeled tubes continued to label L-arabinose (Ara) and Xyl units of pectin as well as Ara units from amyloglucosidase-treated polysaccharides. When the labeled marker was [1-<sup>14</sup>C]Glc, only the control (no added MI) contained [<sup>14</sup>C]Ara. With increasing levels of Glc, only hexosyl units (Glc, Gal, Rha) were labeled (Maiti and Loewus, 1978b). A functional MIOP in lily pollen tubes was the simplest interpretation of these results.

### **3.3.6 Role of phytate-derived MI in pectin and hemicellulose biosynthesis**

Another approach to the question of MIOP functionality was taken by examining utilization of [2-<sup>3</sup>H]MI by wheat kernels either by imbibition during

germination or by injection into partially digested endosperm of 72 h seedlings. Results indicated that MI reserves (phytate) within the caryopsis provided a significant portion of the carbon requirements for pectin and hemicellulose biosynthesis during germination (Maiti and Loewus, 1978a,b). Imbibition of an aqueous solution of MMO at 40  $\mu\text{g}$  per caryopsis delayed germination by 50 h. At 400  $\mu\text{g}$  of MMO per caryopsis, only 14% of the kernels germinated in 94 h. If MI was included in the germination medium, it partially reversed the inhibition. MMO failed to alter the relative distribution of  $^3\text{H}$  from MI into galacturonyl, arabinosyl, or xylosyl units of pectin and hemicellulose although the total amount of  $^3\text{H}$  incorporated diminished.

### **3.3.7 Comparative study of $^3\text{H}$ -labeled *myo*- and *scyllo*-inositol metabolism in maturing wheat**

In this study,  $[2\text{-}^3\text{H}]\text{MI}$  or *scyllo*-[randomly positioned- $^3\text{H}$ ]inositol ( $[\text{R-}^3\text{H}]\text{SI}$ ) was injected into hollow peduncles of post-anthesis developing wheat spikes. There was rapid translocation and accumulation of  $^3\text{H}$  in developing kernels. In the case of  $[2\text{-}^3\text{H}]\text{MI}$ , 50–60% of the  $^3\text{H}$  from MI was found in cell wall polysaccharides that were recovered from the stem-region of the injection. That portion translocated to kernels was recovered in cell wall polysaccharides, phytate, galactinol, and MI. In the case of  $[\text{R-}^3\text{H}]\text{SI}$ , most of the  $^3\text{H}$  was translocated to kernels where it accumulated as  $[\text{R-}^3\text{H}]\text{SI}$  and *O*- $\alpha$ -galactosyl-SI. No  $^3\text{H}$  from  $[\text{R-}^3\text{H}]\text{SI}$  was found in cell wall polysaccharides or phytate (Sasaki and Loewus, 1980).

When kernels from labeled plants were germinated, most of the tritiated galactinol or galactosyl-SI hydrolyzed within 1 day. Phytate from  $[2\text{-}^3\text{H}]\text{MI}$ -labeled kernels released free  $[2\text{-}^3\text{H}]\text{MI}$  over several days.  $[2\text{-}^3\text{H}]\text{MI}$ -labeled cell wall polysaccharides were also degraded during germination and their sugar residues were reutilized for new cell wall formation in developing shoot and roots. Most of the newly released  $[^3\text{H}]\text{MI}$  from phytate and galactinol recycled over the MIOP into pentosyl residues of cell wall polysaccharides (Sasaki and Loewus, 1982).

### **3.3.8 The MIOP and glucogenesis**

Cyclization of Glc-6-P to MI-1-P (Step 1, Figure 1) is essentially irreversible (Loewus and Loewus, 1974). Subsequent MIOP steps leading to UDP-GluUA and its metabolic conversion to uronosyl and pentosyl components of cell wall polysaccharides offer no direct metabolic routes to glycosyl products such as starch. Nevertheless, prolonged tube growth of lily pollen in media containing  $[2\text{-}^3\text{H}]\text{MI}$  led to accumulation of a significant fraction of  $^3\text{H}$  in  $^3\text{H}_2\text{O}$  and starch-derived  $[^3\text{H}]\text{Glc}$ . Pollen germinated in media containing  $[2\text{-}^{14}\text{C}]\text{MI}$  stored much less  $^{14}\text{C}$  in starch (Rosenfield and Loewus, 1975). Both  $[2\text{-}^3\text{H}]\text{MI}$  and  $[2\text{-}^{14}\text{C}]\text{MI}$  were converted to C5-labeled pentosyl products without

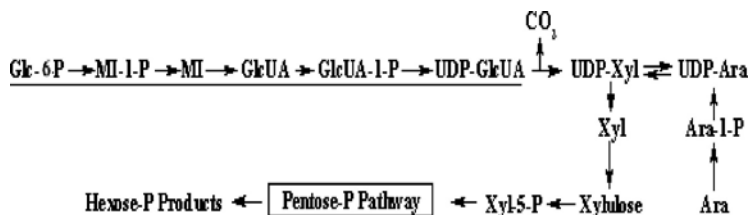


Figure 2. Metabolic route from MI to starch-derived Glc via the MIOP (reaction sequence underlined).

redistribution or loss of label during passage over the MIOP. It was during subsequent interconversion of pentose to hexose that exchange of  $^3\text{H}$  with water and redistribution of  $^{14}\text{C}$  within starch-derived Glc occurred. This could be demonstrated by growing lily pollen tubes in media containing  $[1-^{14}\text{C}]\text{Ara}$ ,  $[5-^{14}\text{C}]\text{Ara}$ , or  $[5-^3\text{H}]\text{Xyl}$ . Metabolic pathways (Figure 2) incorporating these pentoses into cell wall polysaccharides or into starch via pentose-P/hexose-P interconversion occurred beyond the MIOP (Rosenfield and Loewus, 1978a,b; Rosenfield *et al.*, 1978).

### 3.3.9 MI and xylan biosynthesis

Non-cellulosic polysaccharides are intimately associated with cellulose in plants and often account for over half of the polysaccharide content of the primary cell wall, much of it as pectin and xyloglucan (O'Neill and York, 2003). In non-graminaceous plants, xyloglucan, and xylan are the most abundant hemicellulosic polysaccharides of mature wood, as much as 20–30% of dry wall-mass (Mellerowicz *et al.*, 2001). UDP-GlcUA, sole source of UDP-Xyl for xylan biosynthesis, is the ultimate product common to both the MIOP and SNOP. In the latter pathway, UDP-GlcUA is produced by UDP-Glc dehydrogenase, an enzyme strongly inhibited by UDP-Xyl, whereas in the MIOP this product is formed by UDP-GlcUA-1-P uridylyltransferase which is not inhibited by UDP-Xyl. In the event that free MI or a metabolically addressable source of MI is available, the MIOP might provide sufficient UDP-Xyl to limit oxidation of UDP-Glc via the SNOP, in essence, redirecting utilization of UDP-Glc toward biosynthesis of UDP-glycans while the MIOP continues to produce UDP-GlcUA for xylan biosynthesis. Studies described by Imai and associates support such a scheme (Imai and Terashima, 1991, 1992; Imai *et al.*, 1997, 1998, 1999). They found that immature, differentiating, xylem tissue of magnolia produced xylan-containing  $[^3\text{H}]\text{Xyl}$ -residues within 24 h after administration of  $[2-^3\text{H}]\text{MI}$  to growing stems and this product was selectively retained as xylan in mature cell walls 5 months later (Imai *et al.*, 1999).

#### 4. UDP-GlcUA: PRECURSOR OF URONOSYL AND PENTOSYL COMPONENTS IN CELL WALL POLYSACCHARIDES

In view of a potential regulatory role for the MIOP associated with MI biosynthesis or intermediates leading to UDP-GluUA via the MIOP, renewed attention needs to be given to these processes (Kärkönen, 2005). They include use of MIPS mutants that alter free MI availability, hormonal effects on growth or development, modifications of enzymes involved in the MIOP, use of specific inhibitors such as MMO, and application of a host of newly developed tools of molecular biology. A model of such approaches is found in evidence for coexistence and use of both MIOP and SNOP during UDP-GluUA formation in young *Arabidopsis* seedlings (Kanter *et al.*, 2005; Seitz *et al.*, 2000). Moreover, their data suggest that each pathway exhibits temporal and spatial regulatory responses reflecting unique qualities of that pathway.

##### 4.1 Effects of plant growth regulators on MI metabolism

Fifteen-day-old suspension cultures of *Acer pseudoplatanus* grown in Murashige and Skoog medium with 3% Glc, 4.4  $\mu\text{M}$  6-benzylaminopurine (BA) and 0.45  $\mu\text{M}$  2,4-dichlorophenoxyacetic acid (2,4-D) readily took up 100 mg/l of [2- $^3\text{H}$ ]MI over 24 h and utilized up to 20% for pectin biosynthesis. Virtually all of this  $^3\text{H}$  was recovered in galacturonosyl and pentosyl residues. Increasing the BA level 10-fold drastically blocked [2- $^3\text{H}$ ]MI uptake and little was available for pectin biosynthesis. Increasing the 2,4-D level 10-fold had little or no effect on [2- $^3\text{H}$ ]MI uptake but did diminish the amount of  $^3\text{H}$  appearing in pectin (Verma *et al.*, 1976).

Exogenously applied abscisic acid (ABA) suppressed elongation of squash hypocotyl segments and inhibited incorporation of [1- $^{14}\text{C}$ ]Glc and [2- $^3\text{H}$ ]MI into cell wall fractions (Wakabayashi *et al.*, 1989). In the absence of ABA, the extent of incorporation of [1- $^{14}\text{C}$ ]Glc was much greater than that of [2- $^3\text{H}$ ]MI but label from the former appeared uniformly in hexosyl, pentosyl, and uronosyl residues while the latter was limited to just pentosyl and uronosyl residues of pectin and hemicellulose B suggesting that the MIOP was involved.

In studies on ABA-promoted responses in the duckweed, *Spirodela polyrrhiza*, it was found that such treatment activates a developmental pathway culminating in formation of turions, modified resting-state fronds distinguishable from vegetative fronds due to thicker walls, absence of aerenchyma, and accumulation of anthocyanin, MI, starch, and phytic acid (Flores and Smart, 2000; Smart and Fleming, 1993; Smart and Flores, 1997; Smart and Trewavas, 1983). The last-mentioned citation found, in addition to phytic acid and its expected precursors, three novel plant inositol phosphates, two of which were diphosphoinositol pentakis and hexakis phosphates (InsP<sub>7</sub> and InsP<sub>8</sub>), putative functional high-energy intermediates (Safrany *et al.*, 1999; Saiardi *et al.*,

2002). Continued exposure to ABA led to less  $^3\text{H}$  in turion cell walls relative to that in inositol polyphosphates. It is tempting to speculate that diverse regulatory functions involving ABA are involved. These might include one or more of the following: altering contributions from SNOP or MIOP to UDP-GlcUA, regulating phosphorylation of  $[2\text{-}^3\text{H}]\text{MI}$  to  $[2\text{-}^3\text{H}]\text{MI-1-P}$  (initial step in production of polyphosphorylated MIs) or altering demands on the free MI pool that must supply a host of other MI requirements involved in turion development. Only further study of the broad effects of ABA on turion development will resolve the current impetus.

#### **4.2 UDP-GlcUA decarboxylase (EC 4.1.1.35)**

UDP-GlcUA decarboxylase (UDP-GlcUADase) which catalyzes the conversion of UDP-GlcUA to UDP-xylose (UDP-Xyl) has been purified and cloned from a fungus, *C. neoformans* (Bar-Peled *et al.*, 2001), pea seedlings, *Pisum sativum* L. (Kobayashi *et al.*, 2002), *Arabidopsis* (Harper and Bar-Peled, 2002), and immature rice seeds, *Oryza sativa* cv. Nipponbare (Suzuki *et al.*, 2003). It has also been purified and characterized from differentiating tobacco cells (*Nicotiana tabacum* L.) where its 87-kDa isoform is localized to cytoplasm (Wheatley *et al.*, 2002). As Reiter and Vanzin (2001) point out, UDP-GlcUADase represents a major branch point in the biosynthesis of UDP-sugars, involving epimerization, decarboxylation, and rearrangement reactions; the source of uronosyl and pentosyl residues found in pectin and hemicellulose as well as products such as free Xyl (Loewus *et al.*, 1962; Rosenfield and Loewus, 1978a), apiosyl residues of cell wall polysaccharide from *Lemna* and apiin (Roberts *et al.*, 1967b). While these reactions are beyond discussion of processes dealt with in this chapter, it is well to consider their competitive roles apropos their common source. As noted above (Section 3.3.9), feedback inhibition of UDP-Glc dehydrogenase by UDP-Xyl is one such instance.

#### **4.3 Putative role of MI as a source of D-galacturonate for AsA biosynthesis via the MIOP**

Interest in AsA biosynthesis was greatly motivated by comparative studies on plants and AsA-synthesizing animals (Isherwood *et al.*, 1954). Subsequent findings stemming from radioisotopic experiments suggested that two pathways to AsA occurred in plants, one referred to as the “non-inversion” or “direct” route, since it conserved the original carbon chain sequence of Glc, appeared to be the biosynthetic pathway while the other, the “inversion” route, seemingly a salvage pathway (see Section 2). Subsequently, enzymatic and molecular evidence were obtained for the “direct” route, now referred to as the “D-mannose/L-galactose” pathway (Laing *et al.*, 2004; Running *et al.*, 2003; Smirnov *et al.*, 2004; Wheeler *et al.*, 1998). The putative “inversion”

route (Loewus, 1963) gained fresh interest when Agius *et al.* (2000) isolated and characterized GalUR, a gene from strawberry encoding a NADPH-dependent D-galacturonate reductase. They examined strawberry fruit over the full range of development and found expression of GalUR to be positively correlated to AsA content with highest levels of expression in the fully ripe berry where hydrolyzed pectic products rich in D-galacturonic acid (GalUA) occur.

In another significant development, evidence was obtained that GDP-Man 3',5'-epimerase produces novel intermediates, GDP-L-gulose, (Wolucka and Van Montagu, 2003) as well as GDP-L-Gal (Smirnoff *et al.*, 2004) and both of these nucleotides function as intermediates in AsA biosynthesis in plants. Over 40 years ago, Calvin's group reported the isolation of a diphosphate ester of 2-keto-L-gulonic acid from *Chlorella pyrenoidosa* metabolizing  $^{14}\text{CO}_2$  in the light and speculated on its possible role in AsA biosynthesis (Moses *et al.*, 1962). Detached bean apices and ripening strawberry fruits stem-fed aqueous solutions of L-gulono- or L-galactono-1,4-lactone readily convert these compounds to AsA (Baig *et al.*, 1970). At the time, three possibilities were offered for these results: (1) utilization of either substrate by a single oxidizing enzyme, (2) existence of separate oxidizing enzymes for each substrate, or (3) a two-step process wherein epimerization at carbon 3 precedes oxidation. Wolucka and Van Montagu's findings now suggest that both L-gulono- and L-galactono-1,4-lactone may be present as intermediates and that oxidation to AsA is a site-specific process. Jain and Nessler (2000) found that constitutive expression of L-gulonolactone oxidase cDNA in *vtc1-1* mutant *A. thaliana* plants increased AsA content twofold (Conklin, 2001). In an extension of this finding, L-gulonolactone oxidase constructs were also expressed in other *A. thaliana* lines defective in AsA production but unrelated to the defect unique to the *vtc1-1* mutant. All five *vtc* mutant lines that were tested rescued AsA content, equal or higher than that of wild-type plants, suggesting existence of alternative AsA pathways (Radzio *et al.*, 2003).

As noted earlier, experiments leading to a bacterially expressed recombinant protein from chromosome 4 of *Arabidopsis* with the properties of MIOase have provided opportunity to test the full potential for AsA biosynthesis through the "inversion" pathway (Lorence *et al.*, 2004). It remains to be determined whether this route is ancillary to the D-mannose/L-galactose (alternatively, L-gulose) pathway or is a major source of AsA via MIOase in plants.

## 5. CONCERNING A METABOLIC ROLE FOR MYO-INOSITOL IN PLANT CELL WALL BIOGENESIS

This synoptic review of studies involving MI metabolism as applied to formation of uronosyl and pentosyl residues of cell wall polysaccharides is intended

as a reminder to those involved in research on cell wall structure and function of need to give closer scrutiny to the MIOP as an alternative pathway. A recent book (Rose, 2003), prefaced as "...written at professional and reference level...", dismisses the MIOP with a single reference (Doblin *et al.*, 2003: page 205) to a review (Feingold and Avigad, 1980) that was prepared over 24 years ago. Gibeaut (2000) cites Schlüpmann *et al.* (1994) as an example of a sucrose requirement for normal *in vitro* growth of *Nicotiana* pollen but fails to mention the findings of Dickinson (1965, 1967, 1978) as regards tolerance toward pentaerythritol as non-metabolizable osmoticum for normal *in vitro* growth of *Lilium* pollen in the absence of sucrose. Under such growth conditions, *Lilium* pollen utilized [ $^{14}\text{C}$ ]MI for tube wall pectic substance biosynthesis for periods up to 8 h (Kroh and Loewus, 1968). Gibeaut cited a study on the uptake and metabolism of [ $1\text{-}^{14}\text{C}$ ]Glc versus [ $2\text{-}^3\text{H}$ ]MI by etiolated squash hypocotyl segments (Wakabayashi *et al.*, 1989) as evidence for selective cell wall labeling by Glc, ignoring the fact that they were comparing labeling processes in intact pollen tube germination/elongation to that of elongation of hypocotyl segments from squash.

Reiter and Vanzin (2001) and Mellerowicz *et al.* (2001) offer refreshing excursions into an emerging viewpoint that includes both the SNOP and MIOP contributions to UDP-GluUA biosynthesis and subsequent steps of epimerization, decarboxylation and rearrangements to furnish UDP-Gal, UDP-Xyl, UDP-Ara, and UDP-apiiose (Burget *et al.*, 2003; Mølhø *et al.*, 2003). Hopefully, the pioneering efforts of Tenhaken and his colleagues (Kanter *et al.*, 2005; Seitz *et al.*, 2000; Tenhaken and Thulke, 1996) will continue to uncover new molecular and biochemical details on the inter-relationships of the MIOP and SNOP and Nessler's group (Jain and Nessler, 2000; Lorence *et al.*, 2004; Radzio *et al.*, 2003) will extend their studies on the biosynthesis of AsA and its relationship to MI metabolism.

## 6. **MYO-INOSITOL AS POTENTIAL PRECURSOR OF L-TARTRATE AND OXALATE (BREAKDOWN PRODUCTS OF L-ASCORBATE)**

Experiments have yet to be performed which tie production of oxalate and L-threonate/L-tartrate in plants to a specific pathway of AsA biosynthesis (Bánhegyi and Loewus, 2004) but the ease with which AsA is synthesized in phloem and transported via vascular processes from source to sink (Fransceshi and Tarlyn, 2002; Hancock *et al.*, 2003; Tedone *et al.*, 2004) suggests that MI-linked AsA biosynthesis (Lorence *et al.* 2004) may play a significant role in this process. It is of interest to note that an invertebrate, the marine demosponge, *Chondriosia reniformis*, which sheds substantial amounts of crystalline calcium oxalate, contains the same level of AsA as is found in plants (Cerrano *et al.*, 1999). The biosynthetic pathway of AsA in this organism and its putative role as a precursor of oxalate have yet to be determined.



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