

ABSTRACT

CHUANG, LING. Phosphorylation is An On/Off Switch for 5-Hydroxyconiferaldehyde *O*-Methyltransferase Activity in Monolignol Biosynthesis of *Populus trichocarpa*. (Under the direction of Vincent L. Chiang).

To meet environmental and developmental needs, the monolignol biosynthetic pathway for lignification in plant cell walls is regulated by complex mechanisms involving transcriptional, post-transcriptional, and metabolic controls. However, post-translational modification by protein phosphorylation had not been demonstrated in the regulation of monolignol biosynthesis. In this research, the discovery of phosphorylation mediated on/off regulation of enzyme activity for 5-hydroxyconiferaldehyde *O*-methyltransferase 2 (PtrAldOMT2), an enzyme central to monolignol biosynthesis for lignification in stem differentiating xylem (SDX) of *Populus trichocarpa*, was presented. Global shotgun proteomic analysis of *P. trichocarpa* SDX protein fractions identified PtrAldOMT2 mono-phosphorylation at Ser¹²³ or Ser¹²⁵ *in vivo*. *P. trichocarpa* SDX protein extracts phosphorylated recombinant PtrAldOMT2 and turned off the enzyme activity. ATP/Mn²⁺-activated phosphorylation of SDX protein extracts reduced the endogenous SDX PtrAldOMT2 activity by approximately 60%, and dephosphorylation fully restored the activity. Phosphorylation-site mutagenesis verified the PtrAldOMT2 phosphorylation at Ser¹²³ or Ser¹²⁵, and confirmed the functional importance of these phosphorylation sites for *O*-methyltransferase activity. The Ser¹²³/Ser¹²⁵ phosphorylation sites are conserved across 98% of AldOMTs from 46 diverse plant species. The reversible mono-phosphorylation at Ser¹²³ or Ser¹²⁵ acting as an on/off switch for the activity of PtrAldOMT2 provides a rapid and energetically efficient mode of regulating PtrAldOMT2 activity for syringyl monolignol biosynthesis, and represents an additional level of control for monolignol biosynthesis in *P. trichocarpa*.

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Phosphorylation is An On/Off Switch for 5-Hydroxyconiferaldehyde *O*-Methyltransferase
Activity in Monolignol Biosynthesis of *Populus trichocarpa*

by
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BIOGRAPHY

I love nature. Biology is cool and complex. As trees are some of the most ancient organisms on earth, to uncover the mysteries under those old brown barks and green leaves through science methodology is worthy of a lifelong interest. Being on the way of seeking truth in nature is the most inspiring way to live my everyday life.

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Chapter 1 Introduction

1.1 Lignin

1.1.1 The lignified plant secondary cell wall

Lignin is one of the most abundant natural polymers on earth. It is exclusively deposited in the secondary cell walls of plants. Plant cells are encased in walls that can contribute to the majority of the plant biomass. Plant cell walls can be categorized into primary and secondary cell walls depending upon their deposition time at cell growth and their state of differentiation (1, 2). Primary walls are synthesized during cytokinesis and modified during cell expansion. They are mainly composed of cellulose, hemicellulose, protein and pectin. Secondary cell walls are synthesized after the cessation of cell expansion in certain cell types (e.g. fibers, vessel cells) and tissues (e.g. xylem, sclerenchyma), and are characterized by the deposition of lignin polymer (2). The amounts and composition of the major wall components, namely cellulose, hemicellulose, and lignin, vary considerably among plant species, cell types (3) and change during growth and response to biotic and abiotic stress (4). The heterogeneous lignin polymer plays an essential role in plant physiology (3). It serves as a matrix around the polysaccharide components of the plant cell walls, providing additional rigidity and compressive strength as well as rendering the walls hydrophobic and water impermeable, in order to transport water and solutes (5). Thus, secondary cell walls provide mechanical strength to support the plant's weight, and facilitate the transport of water and nutrients. The mechanical support and water transport are both essential for terrestrial vascular plants, which may have appear of only after the evolution of lignin biosynthesis (5). In trees, 15-36% of the dry weight

of wood is lignin, to which provides mechanical support and hydrophobic surface for water transport to hundred meters (5).

Secondary cell walls have been important to humans throughout history as a source of timber, paper, textiles and now as a major source of renewable biomass for the production of second generation biofuels (6). Lignin, as a major obstacle in chemical pulping, forage digestibility, and processing of plant biomass to biofuels, has attracted significant research attention. Industries would benefit from more efficient processing of plant cell wall biomass with less lignin or with lignin that is easier to degrade (5).

1.1.2 Monolignol biosynthesis

Lignin is generated by free radical coupling of hydroxycinnamyl alcohols named monolignols: coniferyl alcohol, sinapyl alcohols, and 4-coumaryl alcohol. When introduced in the lignin polymer the corresponding monolignol subunit are named guaiacyl (G), syringyl (S), and 4-hydroxyphenyl (H) units, respectively. The composition of lignin varies between plant species and tissues. In general, lignin from gymnosperms and related species are rich in G units and contains low amounts of H units, whereas dicot lignins are mainly composed of G and S units (7). The biosynthesis of monolignols is initiated from the general phenylpropanoid pathway (8). Several other major classes of plant products in addition to lignin are derived from phenylalanine, including flavonoids, coumarins, stilbenes, and benzoic acid derivatives (9). Monolignols are generated from phenylalanine via a series of enzymatic reactions, catalyzed by 10 families of enzymes (**Fig. 1-1**) (10). The first enzyme of the general phenylpropanoid pathway is phenylalanine ammonia-lyase (PAL) (EC: 4.3.1.5), which catalyzes the deamination of phenylalanine to produce cinnamic acid, a nearly universal intermediate in the formation of a large variety of plant specific phenylpropanoid derivatives.

The hydroxylation steps of the monolignol biosynthesis pathway are catalyzed by cinnamic acid 4-hydroxylase (C4H) (EC: 1.14.13.11), coumaric acid 3-hydroxylase (C3H) (EC: 1.14.14.1), and coniferaldehyde 5-hydroxylase (CAld5H) (EC: 1.14.13), which belong to the cytochrome P450 monooxygenase superfamily, more specifically the CYP73, CYP98 and CYP84 families, respectively. The second enzyme of the general phenylpropanoid pathway, C4H, catalyzes the 4-hydroxylation of cinnamic acid into 4-coumaric acid. C3H catalyzes predominantly the 3-hydroxylation of 4-coumaroyl shikimic acid. The C3H-catalyzed reaction is the first irreversible step toward S and G lignin (11). CAld5H (EC: 1.14.13) catalyzes the 5-hydroxylation primarily of coniferaldehyde, leading to sinapyl alcohol and ultimately, to S lignin (12, 13). 4-Coumaric acid:coenzyme A ligase (4CL) (EC: 6.2.1.12), catalyzes the formation of CoA thioesters of 4-coumaric acid and other 4-hydroxycinnamic acids in a two-step reaction involving the formation of an adenylate intermediate (14). The methylation steps in the monolignol biosynthesis pathway are catalyzed by 5-hydroxyconiferaldehyde *O*-methyltransferase (AldOMT) (EC: 2.1.1.68) and caffeoyl-CoA *O*-methyltransferase (CCoAOMT) (EC: 2.1.1.104) (15). Plants contain a wide variety of S-adenosyl-L-methionine (SAM) dependent *O*-methyltransferases that act on phenylalanine derived substrates during the production of numerous plant secondary compounds in addition to lignin (16). Hydroxycinnamoyl-CoA: shikimate hydroxycinnamoyl transferase (HCT) (EC: 2.3.1.133), an acyltransferase, catalyzes the reactions both immediately preceding and following C3H/C4H (17, 18). HCT catalyzes the transfer of 4-coumaroyl-CoA and caffeoyl-CoA to shikimate for the biosynthesis of corresponding shikimate esters (17). Cinnamoyl-CoA reductase (CCR) (EC:1.2.1.44) catalyzes the conversion of cinnamoyl-CoA esters to their corresponding cinnamaldehydes (19). In the last step, cinnamyl alcohol dehydrogenase (CAD) (EC:

to the cell wall has remained elusive. Recently, an ATP-binding cassette transporter (ABC transporter) acting as a 4-coumaryl alcohol transporter has been identified (20). It is suggested that coniferyl alcohol is transported into the cell wall across the plasma membrane in an ATP-dependant process by an ABC-transporter and is subsequently polymerized by laccases (LACs) and/or peroxidases (PRXs). In contrast, the glycosylated form (e.g., coniferin) is transported into the vacuole for storage (20). The monolignols are oxidized by peroxidases or laccases via oxidative coupling (21, 22). The monolignols are first radicalized, the monomeric radicals then couple to each other or to an existing polymer. The broad substrate specificities and the large gene families of these two classes of oxidases have made it difficult to identify isoforms that are specifically involved in developmental lignification (21, 22). Two theories of lignin polymerization had been developed. The original theory developed by Freudenburg suggested a combinatorial model that monolignols in the extracellular space polymerize with one another in the absence of proteinaceous control, subject only to the supply of monolignols and the usual physical/chemical properties governing chemical reactions (23, 24). The other hypothesis surmises that the lignin monomers are coupled with absolute structural control by proteins bearing arrays of dirigent guiding sites. The proteins responsible for such control have been proposed to contain specific radical binding sites similar to those found in characterized dirigent proteins, and dictates the order of monolignols and intersubunit linkages (25)

1.2 Regulation of Monolignol Biosynthesis

1.2.1 Transcriptional regulation

Regulation of monolignol biosynthesis is complex and involves several different control mechanisms at different levels from genes, transcripts, proteins to the final product-

metabolites. The most extensively investigated regulation mechanism might be the transcriptional regulation network. Genes responsible for synthesis of the different cell wall components are coordinately expressed and under transcriptional regulation. Monolignol biosynthesis is regulated by transcriptional activation and suppression by wood-associated NAC (NAM, ATAF1/2 and CUC2) domain factors, MYB transcription factors and their alternative splice variants (26, 27). The cell wall related transcription factors form a complex network, which tightly regulates cell wall related gene expression including monolignol enzymes. A transcription factor (TF)-directed hierarchical gene regulatory network (hGRNs) for wood formation in *Populus trichocarpa* based on overexpression of a NAC transcription factor in xylem protoplasts has recently been presented (28).

1.2.2 Protein and metabolic level regulation

At the post translational level, monolignol biosynthesis is regulated by protein interactions (29, 30) and a complex network of feed-forward and feed-back enzyme inhibitions (31-33). For protein-protein interaction regulation, it had been shown that the hydroxylases (C4H and C3H) in *Populus trichocarpa* form heterodimeric and heterotrimeric membrane protein complexes that increase the enzymatic efficiency for any of the complexes by 70–6,500 times than that of the individual proteins. The highest increase in efficiency was found for the PtrC4H1/C4H2/C3H3-mediated 4-coumaroyl shikimic acid ester 3-hydroxylation (29). For feed forward and backward metabolic inhibition, 4-coumaroyl and caffeoyl shikimic acids have been identified as competitive and uncompetitive inhibitors of the *Populus trichocarpa* 4-coumaric acid: coenzyme A ligases. 4-Coumaroyl shikimic acid strongly inhibits formation of 4-coumaroyl-CoA and caffeoyl-CoA. Caffeoyl shikimic acid inhibits only formation of 4-coumaroyl-CoA. 4-Coumaroyl and caffeoyl shikimic acids both act as competitive and

uncompetitive inhibitors (34). A predictive kinetic metabolic-flux model of monolignol biosynthetic pathway using *Populus trichocarpa* stem differentiating xylem incorporating reaction and inhibition kinetic parameters of all 21 enzymes with 24 metabolites has been established based on the absolute quantities of all enzymes obtained by mass spectrometry (32).

1.2.3 Post-translational modification

It has been estimated that proteins may undergo at least 125 post translational covalent modifications on their individual amino acids (35). The post translational modification of proteins may affect their functions and thus facilitate or inhibit a wide variety of biological processes. The involvement of post translational modification in the regulation of lignin biosynthesis had long been implied (36), but almost no direct evidence of functional post translational modification involved in monolignol biosynthesis has been found before this work.

Some post translational modification of transcriptional factors involved in secondary cell wall biosynthesis has been identified. In *Pinus taeda*, the lignin activators PtMYB1 and PtMYB4 are phosphorylated by a MAP kinase, PtMAPK6. However, this post-translational modification does not alter DNA binding, but promotes transcriptional activation in yeast (37). The MAPK phosphorylation site is also present in other plant lignin pathway MYBs. The biological importance of such phosphorylation in monolignol biosynthesis remains unknown (38). Another post translational modification, poly(ADP-ribosyl)ation was implied as a functional component in plant responses to biotic stress including callose deposition, lignin deposition, pigment accumulation, and phenylalanine ammonia lyase activity, but it does not disrupt other responses, such as the initial oxidative burst and expression of some early

defense associated genes (39). In *Arabidopsis*, exogenous application of an inhibitor of poly(ADP-Rib) polymerase can block lignin deposition, which is otherwise induced as one of the innate immune responses by microbe-associated molecular patterns, such as bacterial flagellin (39).

The first enzyme in monolignol biosynthesis, PAL, which is also the entry point that direct the primary metabolism into general phenylpropanoid secondary metabolism, was identified as a phosphoprotein decades ago because degradation products of elicitor induced French bean PAL could be radiolabelled by [³²P] orthophosphate *in vivo* (40). Allwood et al. (41) subsequently showed that a synthetic peptide of PAL and a recombinant poplar PAL could be phosphorylated by protein fractions of elicitor induced French bean cell suspension cultures. The same synthetic peptide and poplar recombinant PAL was later shown to be phosphorylated by an *Arabidopsis thaliana* calcium dependent kinase (AtCPK1) (42, 43), but PAL catalytic efficiency was unaffected by this phosphorylation (41). Instead, the phosphorylation was predicted to mark particular PAL subunits for turnover, or to target them for specific subcellular compartments (41, 42). No knowledge of protein phosphorylation was presented for the remaining monolignol biosynthetic enzymes. Given that protein phosphorylation is one of the most widespread types of post-translational protein modification and due to the involvement of protein phosphorylation in lignin related transcription factors and PAL, it is reasonable to speculate that such modification might occur on one or more other lignin related proteins.

1.3 Protein Phosphorylation in Plants

1.3.1. Phosphoprotein detection and phosphoproteomic analysis

One of the most extensively studied post translational modifications is reversible protein phosphorylation. The mechanism of protein phosphorylation regulates biological processes by switching enzyme activities on and off. It was first discovered in a study that elucidated the complex hormonal regulation of skeletal muscle glycogen phosphorylase by Edwin Krebs and Edmond Fisher in 1950 (44). The incorporation of radiolabeled ATP³² into target proteins/peptides for phosphoprotein identification was developed at that time and has been used ever since (44). The involvement of reversible protein phosphorylation based regulation for biological mechanisms in both eukaryotes and prokaryotes has been studied extensively since then. In addition to radiolabeled ATP, several tools have been developed to identify and characterize phosphoproteins, for example, phosphoprotein specific immunodetection or phosphate group specific staining such as Pro-Q diamond staining (45). However, only the most abundant phosphoproteins can be identified by these 2D-PAGE based methods if no prior phosphoprotein enrichment is employed (46). One of the phosphate group specific compounds proposed in 2006 and used to identify phosphoproteins and their phosphorylation level (i.e. number of phosphorylation sites on a single protein) is Phos-tag (47). Phos-tag is a functional compound that specifically binds to phosphorylated ions and can be attached to acrylamide in a PAGE system. In Phos-tag PAGE, proteins with phosphate groups interact with Phos-tag and decrease their migration speed in PAGE, therefore the phosphorylated and non-phosphorylated counterparts are separated. The migration speed of proteins in Phos-tag gel also varies by their degree of phosphorylation, the more phosphate groups a protein has

the slower it moves in the gel, so the same protein with different phosphorylation levels can be separated and distinguished (47).

Recently, the improvement of mass spectrometric analysis instrumentation and the development of phosphopeptide enrichment have facilitated large scale phosphoproteomic analysis. High-throughput identification of phosphoproteins and the sequencing of phosphorylation sites has become more accessible. The general processes involved in a MS-based phosphoproteomics are sample preparation, protein fragmentation/digestion, phosphoprotein or phosphopeptide enrichment, LC-MS/MS analysis for data acquisition, and phosphoprotein/peptide annotation/identification (48). The phosphoprotein/peptide enrichment steps are necessary in phosphoproteomics to minimize the ion suppression effect from non-phosphorylated counterparts. In addition to MS resolution and accuracy, phosphopeptide enrichment is one of the most critical steps in phosphoproteomic analysis. A number of techniques have been developed to preferentially enrich phosphopeptides from a proteolytic mixture. Common strategies include immobilized-metal affinity chromatography (IMAC), which exploits the high affinity of a phosphate group to cations such as Zn^{2+} , Fe^{3+} , and Ga^{3+} (49). Other enrichment methods include metal oxide affinity chromatography (MOAC), Phos-tag chromatography, or pre-fractionation by ion exchange chromatography (SCX and SAX), hydrophilic interaction liquid chromatography (HILIC) and electrostatic repulsion hydrophilic interaction chromatography (ERLIC), polymer based metal ion affinity capture (PolyMAC), hydroxyapatite chromatography, enrichment by chemical modification, and phosphopeptide precipitation (48). Following enrichment, phosphopeptides are injected into LC-MS/MS for data acquisition (46). For phosphoprotein/peptide identification in shotgun analysis, a well annotated genome is required (data dependent analysis) (50).

Though MS-based phosphoproteomics has matured within the last decade into a methodology useful for addressing biological questions in a large-scale, nonhypothesis-driven fashion, the current workflows still leave plenty of room for improvement to approach complete coverage and accuracy of the phosphoproteome (51). The bias in each stage caused by sample complexity, different phosphopeptide enrichment methods, tryptic digestion, and peptide identification/ annotation, still limits the coverage of the phosphoproteome and produces possibility of false discovery for certain phosphoproteins. Thus, technical/ biological replicates, followed by different biochemical methods to confirm and characterize the phosphorylation (level) of specific certain proteins is still needed.

1.3.2 Protein phosphorylation based regulation in plants

Protein phosphorylation had long been implicated in regulating vast numbers of cellular processes in plants through regulating protein activity, location, stability or interactions (52). Due to the MS-based, vastly improved methods for phosphorylation site mapping, and rapid accumulation of plant genome information, hundreds to thousands of phosphoproteins in plants are now identifiable in samples of moderate complexity (50). With the advent of phosphopeptide enrichment and high-throughput mass spectrometry, large scale global phosphoproteomic analysis has now been applied to 20 plant species, and over 31,000 plant phosphoproteins have been identified (53). Because well annotated genome information is required for shotgun analysis, most plant shotgun phosphoproteomics studies have been performed on Arabidopsis. A pioneering study in 2004 reported 283 phosphopeptides from plasma membrane fractions of Arabidopsis using a two-step method that combined SCX and IMAC phosphopeptide enrichment (54), since then the numbers of phosphopeptides identified in Arabidopsis cultured cells and leaves has dramatically increased with the improvement of

phosphopeptide enrichment methods and LC-MS instrumentation (55-57). In 2008, comprehensive phosphoprotein profiling revealed 2,597 phosphopeptides from 1,346 phosphoproteins in cultured Arabidopsis cells. Tyrosine phosphorylation had been thought to be of minor extent in plants until the distribution of phosphoserine, phosphothreonine, and phosphotyrosine sites, which was 85.0, 10.7, and 4.3% , were revealed in this study (58). Using a two-step phosphopeptide enrichment method, 3,029 phosphopeptides from cell lysates of Arabidopsis seedlings were identified in 2009 (50), and 5,143 phosphopeptides were identified from Arabidopsis cell lysates in a comparative phosphoproteome analysis between Arabidopsis, rice (*Oryza sativa*) and *Medicago truncatula* in 2010. This comparative phosphoproteomic study revealed that more than 50% of the phosphoproteins identified in rice and Arabidopsis, which possessed ortholog(s), had an orthologous phosphoprotein in the other species. Moreover, nearly half of the phosphorylated orthologous pairs were phosphorylated at equivalent sites. These data validated the conserved regulatory mechanisms based on phosphorylation in plants (59). Rapid accumulation of plant genome information has allowed analysis of the phosphoproteomes of other plant species. For example, 3,457 phosphopeptides were identified from *Medicago truncatula* roots by Grimsrud et al. in 2010 (60). For monocot species, 6,919 phosphopeptides were identified from rice cells (*Oryza sativa japonica* ‘Nipponbare’) (59); Liu et al. identified 104 phosphopeptides from 94 phosphoproteins in the leaves of *Populus trichocarpa* (61).

Recent phosphoproteomic analysis methodology and techniques largely improve the knowledge in the mechanisms of important signaling systems like reactive oxygen species (ROS), an integral role as signaling molecules in the regulation of numerous biological processes such as growth, development, and responses to biotic and/or abiotic stimuli in plants

(62); abscisic acid (ABA), a phytohormone that regulates diverse plant processes, including seed germination and the response to dehydration, a comparative analyses between wild-type and *srk2dei* triple mutant plants treated under different conditions to identify SNF1-related protein kinase 2 (SnRK2)-dependent phosphoproteins was performed (63-65). The role of protein phosphorylation has shed light on stress sensing like heat (66), cold (67), pathogens (68), circadian clock regulation (69), cell development(68), and photosynthesis (70, 71) in plants.

Although the function of most of these sites is unknown, and some of them may have no discernible biological function other than serving as “a phosphorylation sink” that buffers superfluous kinase activity (72), in general, phosphorylation can regulate protein activities on several levels: (1) as a structural element for correct protein folding (73); (2) by inducing conformational changes to increase or inhibit enzymatic activity; (3) by serving as docking sites for the inducible assembly of complexes with other proteins; (4) as recognition signals for further modification, such as ubiquitination (74); and (5) by changing subcellular locations (75).

Numerous protein phosphorylation studies in plants had been conducted, however, most focused on upstream of the signaling pathway (top down study). Few protein phosphorylation studies directly focused on how protein phosphorylation directly affects the enzymes in a pathway, which are most directly involved in physiological function and phenotype. Here, the role of protein phosphorylation of a monolignol enzyme in *Populus trichocarpa*, PtrAldOMT2, with a central role in S lignin biosynthesis will be extensively studied. Based on the knowledge of the phosphorylation function in the enzyme, a bottom up study could be conducted in the

future to characterize the PtrAldOMT targeted kinases and to investigate association with environmental or developmental stimuli.

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Chapter 2 Phosphoproteomic Analysis of Monolignol Enzymes in *Populus trichocarpa*

2.1 Introduction

Lignin is one of the most abundant biological polymers on the planet (1, 3). This phenolic polymer is deposited in secondary cell walls of vascular plants, and confers hydrophobicity for water transport, compressive strength, and resistance to pests and pathogens (1, 4, 5). Lignin is formed by the free-radical polymerization of monolignols, which are products of the highly regulated monolignol biosynthetic pathway. Monolignol biosynthesis is regulated by transcriptional activation and suppression by wood-associated NAC (NAM, ATAF1/2 and CUC2) domain factors and alternative splicing (6-8). At the metabolic level, monolignol biosynthesis is regulated by protein interactions (9, 10) and a complex network of feed-forward and feed-back enzyme inhibitions (3, 11-13).

Little is known of the potential impact of post-translational modification by protein phosphorylation in monolignol biosynthesis. Protein phosphorylation is one of the most widespread types of post-translational modifications that regulate protein function in response to developmental and environmental stimuli in prokaryotes and eukaryotes (14). Phosphorylation may regulate protein activity, location, stability or interactions. Protein modification by phosphorylation is critical for plants, regulating numerous biological processes such as cellular metabolism, signal transduction, and stress responses (15-17).

The involvement of protein phosphorylation in monolignol biosynthesis was first proposed more than two decades ago, because degradation products of elicitor induced French bean phenylalanine ammonia-lyase (PAL) could be radiolabelled by [³²P] orthophosphate *in*

vivo (18). A synthetic peptide of PAL and a recombinant poplar PAL could be phosphorylated by protein fractions of elicitor induced French bean cell suspension cultures (19), and was later shown to be phosphorylated by an *Arabidopsis thaliana* calcium dependent kinase (AtCPK1) (19, 20). However, PAL catalytic efficiency was unaffected by this phosphorylation (21). No knowledge of protein phosphorylation was presented for the remaining monolignol biosynthetic enzymes.

The objective of phosphoproteomic analysis of monolignol enzymes in *Populus trichocarpa* is to reveal the involvement of protein phosphorylation based regulation of monolignol enzymes in wood formation. *Populus trichocarpa* is a fast-growing woody plant used for pulp and paper for a long time. With its potential for biofuel production and its completely sequenced genome (22), it was selected to be the plant for this study. An LC-MS/MS based shotgun phosphoproteomic analysis of secondary differentiating xylem (SDX) of *Populus trichocarpa* was conducted. The phosphoproteins identified as monolignol enzymes in LC-MS/MS results will be discussed in this chapter. Phos-tag immunodetection was also conducted to confirm the phosphorylation of the monolignol enzymes identified in the phosphoproteomic analysis.

2.2 Materials and Methods

2.2.1 Plant Materials

Populus trichocarpa (genotype: Nisqually-1) was maintained in a greenhouse as described (23). Stem differentiating xylem (SDX) from six-month-old trees was harvested as described (24, 25) for protein and protoplast isolation.

2.2.2 Crude Protein Isolation from *P. trichocarpa* SDX

1.5 g of freshly harvested SDX was ground in liquid nitrogen using a mortar and pestle. The powdered SDX was resuspended in 5 mL of ice cold extraction buffer containing 50 mM Tris-HCl pH 7.5, 20 mM sodium ascorbate, 0.4 M sucrose, 100 mM sodium chloride, 5 mM dithiothreitol, 10% w/w polyvinylpolypyrrolidone, 1 mM phenylmethanesulfonyl fluoride, 9 µg/mL pepstatin, 9 µg/mL leupeptin, 1× PhosSTOP (Roche, Indianapolis, IN) and 10% v/v glycerol. The mixture was homogenized on ice with an electric homogenizer (IKA, Wilmington, NC) for 15 min, filtered through two layers of miracloth (EMD Millipore, Billerica, MA), and centrifuged at 3000 × g for 30 min at 4°C. The supernatant was used immediately for proteomic analysis, immunodetection and enzyme assays.

2.2.3 Filter Aided Sample Preparation and Immobilized Metal Affinity Chromatography for Phosphopeptide Enrichment

750 µg of SDX protein was processed using filter aided sample preparation (FASP) as described previously (26), using two 10 kDa molecular mass cutoff filters (Millipore, Billerica, MA). Samples were digested overnight using modified porcine trypsin with previously optimized parameters for shotgun proteomic analysis (26).

Phosphopeptide enrichment was carried out using iron (III) - immobilized metal affinity chromatography (Fe-IMAC) as described previously (13, 27). An aliquot of 400 µL of nitrilotriacetic acid agarose resin (Qiagen, Venlo, Netherlands) was transferred to a 1.5 mL microcentrifuge tube, centrifuged at 2,000 rpm for 30 sec and the supernatant was removed. This centrifugation was repeated twice. The resin was then washed three times with 1 mL of H₂O, centrifuged for 30 sec and the supernatant was removed. The resin was then washed three times with 1 mL of 1% glacial acetic acid, vortexed and then centrifuged, removing the

supernatant. Finally, 1 mL of 100 mM FeCl₃ in 1% acetic acid was added and the centrifuge tube was wrapped in aluminum foil to prevent exposure to light and placed on a rocker for 4 h. An aliquot of 100 μL of Fe-IMAC slurry was added to a 200 μL gel loader pipette tip (USA Scientific, Ocala, FL) plugged with ultra-high molecular weight polyethylene sheet disks 7-μm pore size and 0.025-inch thickness (Interstate Specialty Products, Sutton MA) to retain the resin. The pipette tip was pre-cut and adapted to fit a standard 5 mL syringe. The resin was then washed twice with 100 μL of 2% acetic acid. 750 μg of digested protein was then dissolved in 100 μL of 2% acetic acid and added to the pipette tip. The flow through was collected and re-loaded onto the pipette tip. The tip was then washed three times with 100 μL 2% acetic acid, then three times with 100 μL of 74/25/1 (100 mM NaCl/Acetonitrile/Glacial Acetic Acid) and finally three times with 100 μL of H₂O. 100 μL of 5% ammonium hydroxide was added and mixed into the pipette tip for at least 2 min and the eluent was collected. This was done three times. The eluent was acidified with 5 μL of 100% formic acid, dried down and dissolved in 100 μL of 0.001% zwittergent immediately prior to analysis.

2.2.4 LC-MS/MS Analysis of Phosphorylated Peptides

Phospho-enriched SDX samples were analyzed in triplicate using a Thermo Scientific EASY nLC II (Thermo Scientific, San Jose, CA) operated in a trap-and-elute configuration and directly coupled to a quadruple orbitrap benchtop mass spectrometer (Q Exactive, Thermo Scientific). An injection of 10 μL was performed to a NanoFlex Chip-LC trap column, 200 μm × 0.5 mm in-line with an analytical column, 75 μm × 15 cm (Eksigent, Redwood City, CA) both packed with Chrom XP C18-CL (3 μm particle size, 120 Å pore size). A 100-min elution was performed at a flow rate of 350 nL/min, utilizing a 5-30% B gradient. Mobile phases A and B were composed of water/acetonitrile/formic acid (98/2/0.2% and 2/98/0.2%,

respectively). Q Exactive instrument parameters, previously optimized for global proteomics were implemented for MS analysis (28). The mass spectrometer was operated in the data-dependent mode in which a full-scan MS (from 400 to 1600 m/z , with resolving power of 70,000 FWHM (full width at half maximum height at 200 m/z) was followed by up to 12 MS/MS scans (top 12 ddMS/MS) with a resolving power of 17,500 FWHM. In addition, two injections using a neutral loss method were performed for phosphorylation 98 Da ($\Delta 48.988$ m/z , $\Delta 35.658$ m/z and $\Delta 24.494$ m/z). An automatic gain control (AGC) target for MS scans was set to 1E6 with a maximum ion injection time (IT) of 60 msec for full MS and an AGC target of 2E4 for MS/MS scans with a maximum IT of 120 msec. Unassigned ions and ions with +1 charge state were rejected from MS/MS isolation and activation. Dynamic exclusion time was set to 30 sec. A normalized collision energy of 27 was used with an isolation window of 2.0 m/z . A capillary voltage of +2.0 kV was used and the temperature was set to 275 °C.

2.2.5 Phos-tag Immunodetection of Phosphorylated PtrAldOMT2

The *in vitro* phosphorylated SDX mixtures were run on a Phosphate Affinity SDS-PAGE containing Acrylamide-pendant Phos-tag (WAKO, Richmond, VA) using 100 V for 3 h at 4 °C. The Phos-tag SDS-PAGE was prepared according to the protocol from WAKO with minor modifications. After electrophoresis, the Phos-tag SDS-PAGE was soaked in 4 mM EDTA containing transfer buffer for 30 min to eliminate manganese ion in the gel, then soaked in transfer buffer without EDTA for an additional 30 min prior to transfer to a PVDF membrane. The transferred PVDF membrane was blocked with 5% non-fat milk in TBST (20 mM Tris pH 8, 150 mM NaCl, 0.05% v/v Tween 20) for 2 h at room temperature. For detection of PtrAldOMT2 recombinant protein, the PVDF membrane was incubated with 1:3000 mouse monoclonal His-tag specific antibody (Sigma Aldrich, St. Louis, MO) in TBST at 4°C

overnight. After washing with TBST, the membrane was incubated with rabbit monoclonal anti-mouse antibody (Promega, Madison, WI) at room temperature for 4 h.

Endogenous PtrAldOMT2 in SDX was immuno-detected by rabbit polyclonal PtrAldOMT2 specific antibody. The secondary antibodies for endogenous PtrAldOMT2 was horseradish peroxidase (HRP)-conjugated anti-mouse antibody. An enhanced chemiluminescent substrate (Thermo, Rockford, IL) was used for signal visualization.

2.3 Results and Discussion

2.3.1 LC-MS/MS Phosphoproteomic Analysis Revealed Monolignol Enzyme Phosphorylation *In Vivo*

The phosphoproteome of *P. trichocarpa* stem differentiating xylem (SDX) was analyzed to investigate *in vivo* post translational protein phosphorylation of monolignol biosynthetic enzymes. Affinity chromatography based phosphopeptide enrichment of tryptic peptides was used to facilitate the identification of phosphoproteins by nano-flow reverse-phase liquid chromatography (LC)-tandem mass spectrometry (MS/MS). Phosphopeptides were enriched based on the affinity of phosphate groups to ferric metal ions. The phosphopeptide enrichment is necessary because phosphorylated peptides are often present in low abundance relative to their more abundant unphosphorylated isoforms (29). Phosphorylation is also a labile modification, capable of undergoing in-source decay during electrospray ionization (29). Moreover, during data dependent acquisition, the mass spectrometer selects peptides for sequencing based on abundance, further biasing the detection of phosphopeptides (29). Due to these analytical challenges, confident identification of phosphorylated peptides is difficult in complex biological sample (29).

The phosphoproteomic analysis of phosphopeptide enriched SDX protein fractions yielded 1439 protein groups and 4836 unique tryptic peptides at a 1% protein false discovery rate (FDR). Of the total enriched proteins and peptides, 1392 proteins (96.7%) and 4728 (97.8%) peptides were phosphorylated, indicating successful enrichment of the phosphopeptides. Two phosphopeptides mapping to PtrAldOMT2 were identified. Mono-phosphorylation at the Ser¹²³ or Ser¹²⁵ residue was identified for the peptides NEDGV(pS)VSPLCLMNQDK and NEDGVSV(pS)PLCLMNQDK (**Fig. 2-1A and B**). These phosphorylated peptides also mapped to one protein other than PtrAldOMT2, which is an ankyrin repeat protein with an unknown function (Gene accession number: *POPTR_0015s15550*). The transcripts of this ankyrin repeat protein were not xylem specific, and its protein was not detected in any of our shotgun proteomic analyses of *P. trichocarpa* SDX (24, 30). In contrast PtrAldOMT2, one of the most abundant proteins in SDX (30), was uniquely identified by other peptides in the same experiment. Therefore, these phosphopeptides identified are most likely specific to PtrAldOMT2. Phosphopeptide for PtrAldOMT2 with doubly phosphorylation of both Ser¹²³ and Ser¹²⁵ was undetected.

Two other phosphorylated monolignol biosynthetic pathway peptides were identified: NGYQNG(pS)SESLCTQR for PtrPAL1 (**Fig. 2-2A**), and IG(pS)FEEELK shared by both PtrPAL4 and PtrPAL5 (**Fig. 2-2B**). PtrPAL4 and PtrPAL5 are xylem specific and abundant, and therefore are the key PALs involved in monolignol biosynthesis (3, 24). PtrPAL1 is shoot-specific, suggesting its involvement in other pathways (24). Early reports of protein phosphorylation in monolignol biosynthesis demonstrated that PAL of hybrid poplar could be phosphorylated *in vitro* (19,20). Subsequently, two poplar phosphoproteomic studies identified 151 and 147 phosphoproteins, in dormant terminal buds of the hybrid *P. simonii* x *P. nigra*

(42) and in differentiating xylem of *P. trichocarpa* (13), respectively. However, none of these phosphoproteins were associated with monolignol biosynthesis. Whether protein phosphorylation affected monolignol biosynthesis of poplar species could not be answered by these previous studies.

Identification of additional phosphoproteins involved with secondary cell wall biosynthesis included five currently known secondary cell wall specific cellulose synthases (PtrCesA7, PtrCesA17, PtrCesA4, PtrCesA8, PtrCesA18) (31), one putative family 43 glycosyltransferase (PtrIRX9), one cytochrome P450 reductase (PtrCPR1), as well as four transcription factors PtrLIM1, PtrLIM2, PtrSND2/3-B1, and PtrSND2/3-B2. Given the clear presence of phosphoproteins involved in lignin, cellulose, and hemicelluloses biosynthesis in SDX of *P. trichocarpa*, these results imply that protein phosphorylation has a role in secondary cell wall formation.

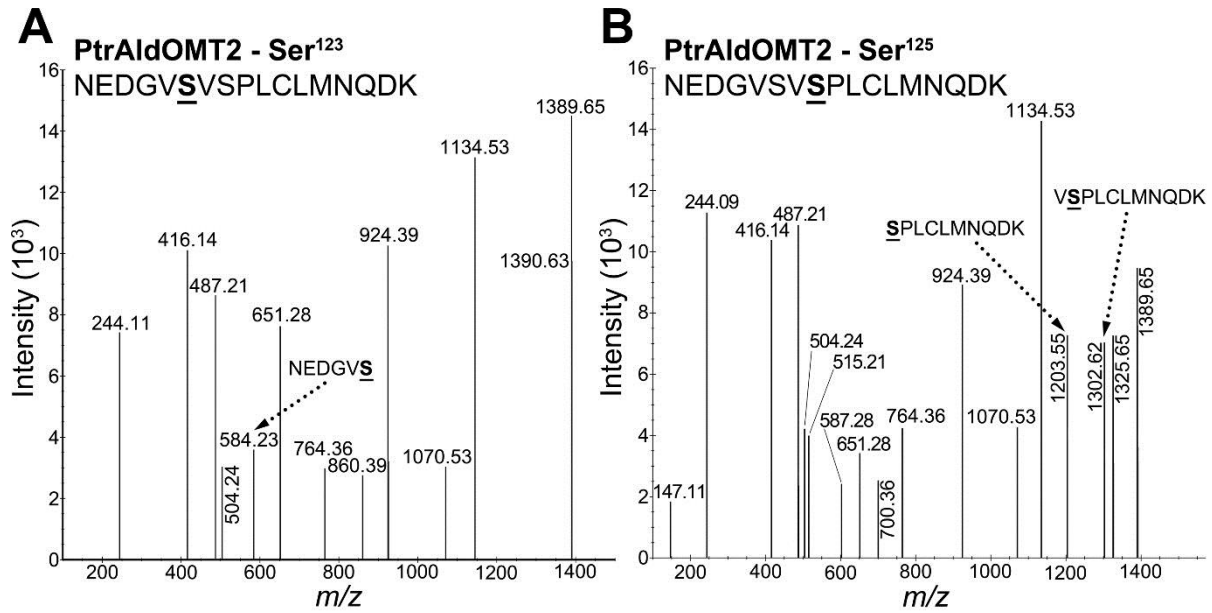


Figure 2-1 PtrAldOMT2 phosphopeptides identified by LC-MS/MS based shotgun proteomic analysis of phosphopeptide enriched *P. trichocarpa* SDX protein fractions. MS spectra: (A) Ser¹²³ and (B) Ser¹²⁵ phosphorylation. Underlined **S** represents phosphorylated serine residues. Fragment ions showing serine phosphorylation are labeled by dashed arrows. Numbers next to peaks denote *m/z* values.

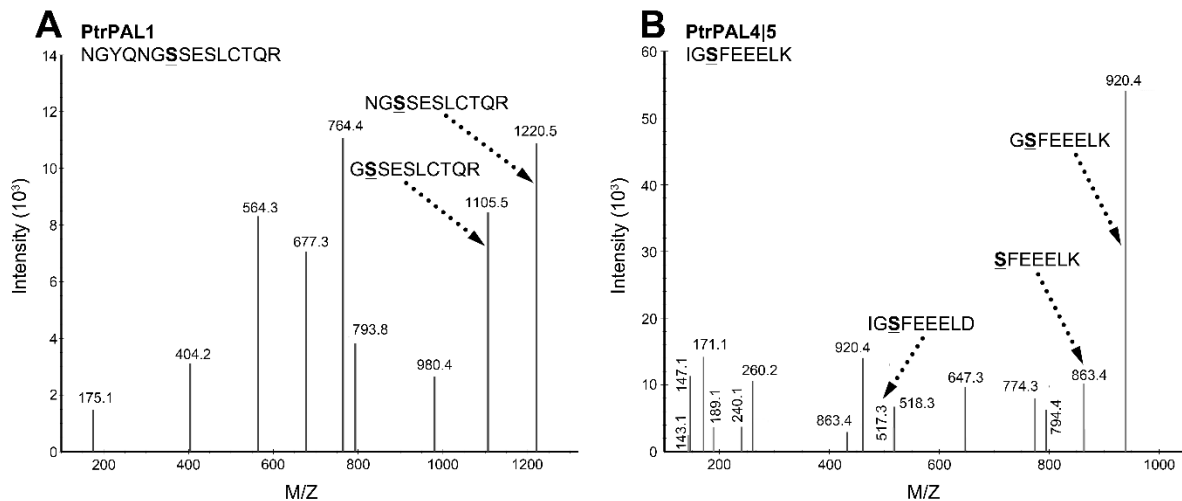


Figure 2-2 Identification of PtrPAL1 and PtrPAL4|5 phosphorylation by LC-MS/MS based shotgun phosphoproteomic analysis of *P. trichocarpa* SDX. (A) MS/MS spectra for phosphorylation of PtrPAL1 in NGYQNGSSES(L)CTQR (B) MS/MS spectra for phosphorylation of PtrPAL4|5 in IGSFEEELK. Red **S** represents the phosphorylated serine residues.

2.3.2 Time Course Analysis of SDX Confirmed Monophosphorylation of PtrAldOMT2

One of the two monolignol enzymes were identified in the phosphoproteomics analysis, PtrAldOMT2, is one of the central enzymes that lead to the formation of S lignin in wood formation. Because the enzyme activity of PAL was not affected by protein phosphorylation, this research focused on how protein phosphorylation affects PtrAldOMT2. To confirm the phosphorylation status of PtrAldOMT2, a time course of *in vitro* phosphorylated SDX was conducted to reveal the phosphorylation level of endogenous PtrAldOMT2. An *in vitro* phosphorylation system was developed to enhance the phosphorylation level of SDX protein bring the abundance of phosphorylated PtrAldOMT2 up to the detection level of Phos-tag acrylamide immunodetection. Phos-tag is a phosphate specific binding compound, while attached to acrylamide in SDS-PAGE, the mobility of phosphoproteins decrease so the phosphorylated proteins are separated from its non-phosphorylated counterparts (32). Proteins with different levels of phosphorylation can also be separated (32). An SDX protein extract was incubated with adenosine triphosphate (ATP) and Mn^{2+} at 37°C to enhance the kinase activity and to phosphorylate target proteins. ATP provides the phosphate group, and divalent metal ions are essential for kinase activity (33). The effects of divalent metal ions on *in vitro* phosphorylation were tested using recombinant protein (Chapter 3). The incubated SDX protein extract aliquots were taken every 30 minutes and analyzed by Phos-tag SDS-PAGE using PtrAldOMT2-specific antibody for detection. The proportion of phosphorylated endogenous PtrAldOMT2 increased with time (**Fig. 3**). The abundance of endogenous phosphorylated PtrAldOMT2 was before the level of immunodetection (**Fig. 2-3**). The phosphorylation level of endogenous PtrAldOMT2 could be enhanced using *in vitro* phosphorylation by SDX endogenous kinases, and the degree of phosphorylation (i.e. number

of phosphophate groups on one protein) was consistent with the mono-phosphorylation results revealed by MS-based analysis.

There was no reduction in the abundance of PtrAldOMT2 during the *in vitro* phosphorylation. Regulating protein-turnover is also a physiological role of phosphorylation in plant metabolism (38). Phosphorylation in plants is known to affect the stability of proteins such as the D1 and D2 proteins of photosystem II (39). Phosphorylation also triggers protein synthesis and degradation, for example DELLA protein regulation of gibberellin-dependent growth processes in plants and their degradation rate is controlled by phosphorylation status (40), and plant TOR kinase stimulates protein synthesis through its impact on phosphorylation of the translational activator S6 kinase (41). In the monolignol biosynthetic pathway, phosphorylation has been implied to affect the stability of recombinant poplar PAL (19). In contrast, there was observed no reduction in PtrAldOMT2 abundance in the ATP/Mn²⁺-activated protein phosphorylation of *P. trichocarpa* SDX protein extracts (**Fig. 2-3**), suggesting that PtrAldOMT2 protein turnover is not regulated by protein phosphorylation.

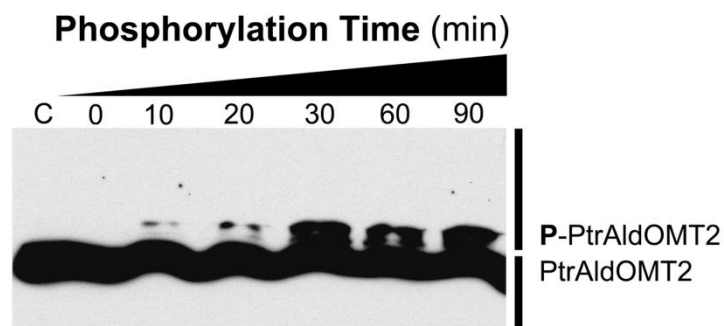


Figure 2-3 *In vitro* phosphorylation of *P. trichocarpa* SDX protein extracts.

ATP/Mn²⁺-activated phosphorylation of *P. trichocarpa* SDX protein extracts, analyzed by Phos-tag SDS-PAGE, and immunodetected using polyclonal anti-PtrAldOMT2 antibody. Control “C” denotes pure PtrAldOMT2-6×His recombinant protein.

2.4 Conclusion

The large scale phosphoproteomic analysis using tandem mass spectrometry of phosphopeptide enriched tryptic fractions identified 1392 specific phosphoproteins in SDX of *P. trichocarpa*. Improved analytical methods and bioinformatics tools, coupled with buffers that inhibit dephosphorylation, have enhanced the sensitivity and coverage of our analysis of the SDX phosphoproteome. Two phosphopeptides mapping to PtrAldOMT2 were identified. Mono-phosphorylation at the Ser¹²³ or Ser¹²⁵ residues was identified for the peptides NEDGV(pS)VSPLCLMNQDK and NEDGVSV(pS)PLCLMNQDK. Time course analysis of phosphorylated SDX confirmed mono-phosphorylation of PtrAldOMT2 and showed that the *in vitro* phosphorylation can enhance the ratio of phosphorylated PtrAldOMT2 compared to its non-phosphorylated counterpart. In addition to PtrAldOMT2, several secondary-cell-wall associated enzymes and transcription factors were found to be phosphorylated. The presence of these phosphoproteins in SDX suggests that protein phosphorylation may regulate the biosynthesis of cellulose, hemicelluloses and lignin, and therefore, secondary cell wall formation.

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Chapter 3 *In Vitro* Phosphorylation of 5-Hydroxyconiferaldehyde *O*-Methyltransferase

3.1 Introduction

In the monolignol biosynthetic pathway, most enzymes are produced in excess of what is required for a normal lignin phenotype (1). This is particularly true for 5-hydroxyconiferaldehyde *O*-methyltransferase 2 (PtrAldOMT2), an enzyme central to syringyl monolignol biosynthesis in *Populus trichocarpa*. A reduction in PtrAldOMT2 abundance to a near complete absence is predicted necessary to modify metabolic-flux for syringyl monolignol biosynthesis (**Fig. 3-1**) (1). 25 gene models encode putative *AldOMTs* in the genome of *P. trichocarpa*. However, only *PtrAldOMT2* is abundantly and specifically expressed in SDX, and therefore is the major *AldOMT* for monolignol biosynthesis in *P. trichocarpa* (2). *PtrAldOMT2* has the most abundant monolignol biosynthetic activity in stem differentiating xylem (SDX) of *P. trichocarpa*, and it is the third most abundant transcript in the whole SDX transcriptome (3). PtrAldOMT2 protein abundance is also the highest of all monolignol biosynthetic enzymes, and accounts for 5.9% of the total SDX proteome (4). Given the abundance of transcript and protein, regulation of PtrAldOMT2 activity by transcriptional control should be energy intensive and slow. Post-translational modifications such as protein phosphorylation may provide a mechanism for regulating PtrAldOMT2 activity without needing to synthesize/degrade protein; thus provide a rapid and energetically efficient mode of regulating *O*-methyltransferase activity for syringyl monolignol biosynthesis.

Protein phosphorylation is one of the most widespread types of post-translational modifications that regulate protein function in response to developmental and environmental stimuli in prokaryotes and eukaryotes (14). Protein phosphorylation regulates numerous

biological processes such as cellular metabolism, signal transduction, and stress responses through regulating protein activity, location, stability or interactions (15-17). Knowledge of how modification by protein phosphorylation regulates the activity of PtrAldOMT2, is needed for a more comprehensive understanding of the regulation of metabolic-flux in monolignol biosynthesis, and should facilitate the genetic engineering of lignin with optimized content and composition to improve the efficiency of processing for pulp and paper production, the digestibility of forage crops or to reduce recalcitrance for more efficient processing of woody biomass for biofuels.

To investigate the mechanism of how protein phosphorylation regulates PtrAldOMT2, phosphorylated PtrAldOMT2 recombinant protein was produced and purified for further functional characterization. Recombinant PtrAldOMT2 protein with His-tag (PtrAldOMT2-6XHis) was constructed and expressed. An *in vitro* phosphorylation system for PtrAldOMT2-6XHis phosphorylation using SDX protein extracts was developed and is described in this chapter. To confirm that the phosphorylation site and status of *in vitro* phosphorylated PtrAldOMT2-6XHis is consistent with endogenous PtrAldOMT2, the phosphorylated protein was re-purified and injected to LC-MS/MS for analysis. A targeted LC-MS/MS analysis was also conducted to measure the enhancement of phosphorylation level of PtrAldOMT2-6XHis after *in vitro* phosphorylation.

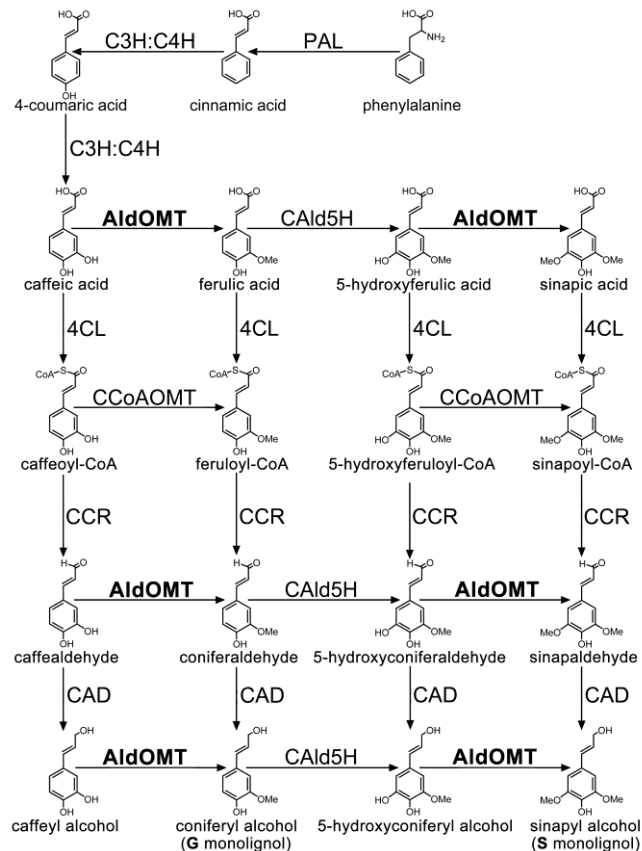


Figure 3-1 The roles of AldOMT in monolignol biosynthesis.

Abbreviations: phenylalanine ammonia-lyase (PAL); cinnamic acid 4-hydroxylase (C4H); coumaric acid 3-hydroxylase (C3H); coniferaldehyde 5-hydroxylase (CAld5H); 4-coumaric acid:CoA ligase (4CL); caffeoyl-CoA *O*-methyltransferase (CCoAOMT); cinnamoyl-CoA reductase (CCR); cinnamyl alcohol dehydrogenase (CAD).

3.2 Materials and Methods

3.2.1 *In Vitro* Phosphorylation of Recombinant PtrAldOMT2

The full-length coding region of the PtrAldOMT2 gene was previously cloned and sequenced (2). Recombinant PtrAldOMT2 protein with a C-terminus 6xHis-tag was expressed in *E.coli* and purified using Ni-NTA (Life technology, Grand Island, NY) His-tag affinity as described by (4).

For analysis of recombinant PtrAldOMT2 phosphorylation, PtrAldOMT2-6×His was mixed into a fresh SDX extract with 5 mM ATP and either 5 mM CaCl₂, 5 mM MgCl₂, or 5 mM MnCl₂, and incubated at 30°C for 3 h. For phosphorylated PtrAldOMT2 enrichment, the *in vitro* phosphorylation assay was conducted by mixing PtrAldOMT2-6×His into fresh SDX extract with 10 mM ATP and 5 mM MnCl₂ then incubated at 30°C for 3 h.

For LC-MS/MS phosphorylated PtrAldOMT2 sample preparation, PtrAldOMT2-6×His was bound to Ni-NTA resin (Life Technology, Carlsbad, CA). The resin bound PtrAldOMT2-6×His was mixed into a protein extract of SDX with 10 mM ATP, 10 mM CaCl₂, 10 mM MgCl₂, and 10 mM MnCl₂ then incubated at 37 °C for 1 h. After washing three times with buffer (50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 60 mM imidazole, 20 mM beta-mercaptoethanol, 10% v/v glycerol), PtrAldOMT2-6×His was recovered with elution buffer (50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 250 mM imidazole, 20 mM beta-mercaptoethanol, 10% v/v glycerol).

3.2.2 LC-MS/MS Analysis of Phosphorylated Peptides

To confirm the phosphorylation site of *in vitro* phosphorylated PtrAldOMT2-6×His, a top 12 ddMS/MS method was performed using the same parameters as performed with phospho-enriched SDX protein extract, and included an inclusion list containing all *m/z* values of peptides identified during shotgun phosphoproteomic analysis of phospho-enriched SDX extracts. Recombinant PtrAldOMT2 samples were processed using the same FASP protocol. Digested recombinant protein samples were then lyophilized and reconstituted immediately prior to LC-MS/MS analysis in 50 µL of 0.001% zwittergent 3-16 (Calbiochem, La Jolla, CA). Phosphorylated PtrAldOMT2-6×His samples were analyzed by 4 injections (3 data

dependent, 1 targeted method). If an m/z contained in the inclusion list is present above a threshold abundance it will be selectively fragmented to obtain an MS2 spectrum. In addition, to ensure consistent monitoring of phosphopeptides of interest and to quantify the site occupancy of the phosphopeptide, a Targeted Selected Ion Monitoring data dependent MS/MS (TSIM-dd MS/MS) method, which increases the sensitivity and reproducibility of phosphopeptide detection by targeted monitoring of specific m/z ratio and selective fragmentation was also implemented. TSIM parameters were analogous to ddMS/MS with the exception of no dynamic exclusion to collect continuous MS/MS spectra when the precursor is above the threshold abundance.

3.2.3 Peptide Identification and Site Occupancy Calculations

Raw files obtained from phospho-enriched SDX protein extract and phosphorylated PtrAldOMT2-6×His samples were converted to “.mgf” files using Proteome Discoverer (Thermo Scientific, San Jose, CA). Resulting “.mgf” files were searched using Mascot Daemon against a concatenated target-reverse *P. trichocarpa* database JGI *P. trichocarpa* v2.2 (Joint Genome Institute, U.S. Department of Energy) containing 45,134 target sequences including corrected sequences recently cloned from *P. trichocarpa* (2). The database search was performed with tryptic *in silico* digestion and allowed a maximum of two missed cleavages on the peptides analyzed from the sequence database. The database search criteria included a fixed modification of cysteine residues for carbamidomethyl modification, and variable modification of other residues to include oxidation of methionine, deamidation of asparagine and glutamine, phosphorylation at serine, tyrosine or threonine for the identification of phosphorylation sites. Peptide precursor mass tolerance was set at 5 ppm, and MS/MS tolerance was set at 0.02 Da for data collected on the Q Exactive. Finally, the search results

(.dat files) obtained from Mascot were imported into ProteoIQ v2.3.08 (Premier Biosoft, Palo Alto, CA) to obtain a final peptide/protein identification list at a protein FDR of 1% and a minimum peptide length of 5 amino acids. Search results (.dat files) were also imported into Skyline v.2.5.0.6079 by building a spectral library where peptides are filtered at a 0.9 probability (5). Site occupancy was calculated using a ratio of the peak area of the phosphorylated peptide to the sum total peak area of the phosphorylated and non-phosphorylated peptides. Peaks were integrated using the ICIS algorithm in Xcalibur v2.2 (Thermo Scientific, San Jose, CA).

3.2.4 Phos-tag Immunodetection of Phosphorylated PtrAldOMT2

The phosphorylated PtrAldOMT2-6×His was desalted with 50 mM Tris-HCl pH 6.8, 50 mM NaCl, 10% v/v glycerol using a 0.5 mL 30 kDa molecular mass cut-off filter (EMD Millipore, Billerica, MA) at 4 °C to remove ATP and salts before Phos-tag SDS-PAGE. The desalted phosphorylated SDX-recombinant protein mixtures were run on a Phosphate Affinity SDS-PAGE containing Acrylamide-pendant Phos-tag (WAKO, Richmond, VA) using 100 V for 3 h at 4 °C. The Phos-tag SDS-PAGE was prepared according to the protocol from WAKO with minor modifications. After electrophoresis, the Phos-tag SDS-PAGE was soaked in 4 mM EDTA containing transfer buffer for 30 min to eliminate manganese ion in the gel then soaked in transfer buffer without EDTA for an additional 30 min prior to transfer to a PVDF membrane. The transferred PVDF membrane was blocked with 5% non-fat milk in TBST (20 mM Tris pH 8, 150 mM NaCl, 0.05% v/v Tween 20) for 2 h at room temperature. For detection of PtrAldOMT2 recombinant protein, the PVDF membrane was incubated with 1:3000 mouse monoclonal His-tag specific antibody (Sigma Aldrich, St. Louis, MO) in TBST at 4°C

overnight. After washing with TBST, the membrane was incubated with rabbit monoclonal anti-mouse antibody (Promega, Madison, WI) at room temperature for 4 h.

Endogenous PtrAldOMT2 in SDX was immuno-detected by rabbit polyclonal PtrAldOMT2 specific antibody and PtrAldOMT2 recombinant protein was detected by mouse monoclonal His-tag specific antibody. The secondary antibodies for endogenous PtrAldOMT2 and PtrAldOMT2-6×His were horseradish peroxidase (HRP)-conjugated anti-mouse and anti-rabbit antibody respectively. The enhanced chemiluminescent (Thermo, Rockford, IL) substrate was used for signal visualization.

3.2.5 Phylogenetic Analysis of PtrAldOMT2 Phosphorylation Sites.

The ClustalW program (6) was used to align the full-length protein sequences of AldOMT from 46 plant species from NCBI (www.ncbi.nlm.nih.gov). The phylogenetic analysis was performed with MEGA version 6 (7) and an unrooted tree was generated using the neighbor-joining method (8).

3.3 Results and Discussion

3.3.1 *P. trichocarpa* SDX Contains Necessary Kinases for Mono-Phosphorylation of PtrAldOMT2

To characterize the physiological impact of PtrAldOMT2 phosphorylation required the isolation of proteins with high levels of this post-translational modification. Because nothing is known of the kinase that phosphorylates PtrAldOMT2, an *in vitro* phosphorylation system was developed using protein extracts of *P. trichocarpa* SDX which contains all the endogenous kinases. Recombinant PtrAldOMT2 with C-terminal His-tag was constructed and produced (**Fig. 3-2A**). To develop this system, various cofactors and substrates associated with protein

phosphorylation were tested, and monitored by Phos-tag SDS-PAGE and western blotting. Phos-tags bind to phosphate groups on proteins and reduce their anionic mobility, therefore enabling the separation of phosphorylated proteins from their unphosphorylated isoform. A time-course analysis of the rate increase of recombinant PtrAldOMT2 phosphorylation by *P. trichocarpa* SDX extract with ATP and divalent metal ions (Mn^{2+} , Mg^{2+} and Ca^{2+}) was first conducted (**Fig. 3-2B**). Relative quantification of phosphorylated PtrAldOMT by densitometric analysis showed that this *in vitro* phosphorylation system enhanced the phosphorylated PtrAldOMT2 to 8% of total PtrAldOMT2 (**Fig. 3-2B**). PtrAldOMT2-6×His phosphorylation was most active when Mn^{2+} and adenosine triphosphate (ATP) were added to the SDX protein extracts (lane 4, **Fig. 3-3**); the addition of Mg^{2+} and ATP to SDX protein extracts only weakly activated PtrAldOMT2-6×His phosphorylation (lane 3, **Fig. 3-3**); no phosphorylation was detected for the addition of Ca^{2+} and ATP to SDX protein extracts, or in reactions without ATP (lanes 1 and 2, **Fig. 3-3**). Mn^{2+} and ATP were thus used for the remaining experiments. The reversibility of PtrAldOMT2 phosphorylation was next tested. Treatment of phosphorylated PtrAldOMT2-6×His with calf intestinal alkaline phosphatase (CIAP) resulted in the complete dephosphorylation of the recombinant protein (lane 5, **Fig. 3-3**). The results showed that recombinant PtrAldOMT2-6×His can be mono-phosphorylated *in vitro*, by incubation in *P. trichocarpa* SDX protein extracts containing ATP and Mn^{2+} (lane 4, **Fig. 3-3**). Complete dephosphorylation of phosphorylated PtrAldOMT2 can be readily achieved, suggesting plasticity in this post-translational modification.

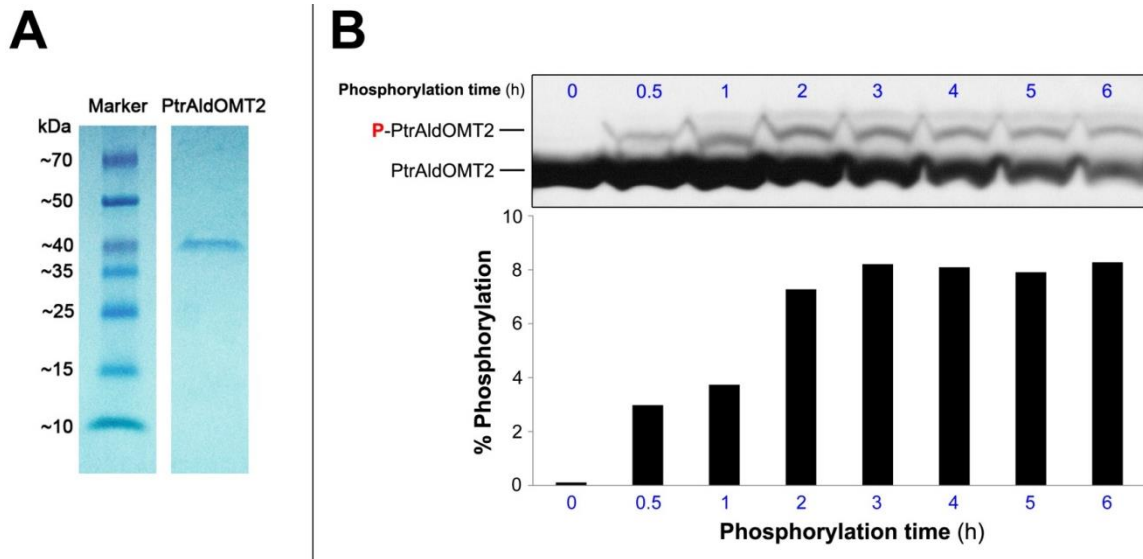


Figure 3-2 Time-course analysis of the rate increase of recombinant PtrAldOMT2 phosphorylation by a *P. trichocarpa* SDX extract.

(A) Purified recombinant PtrAldOMT2 protein stained by Commassie blue stain. (B) Recombinant PtrAldOMT2 protein incubated with *P. trichocarpa* SDX extract and aliquots taken at 0, 0.5, 1, 2, 3, 4, 5, and 6 hours. The aliquots were analyzed by Phos-tag SDS-PAGE and immunodetected by mouse monoclonal anti-His antibody. Relative quantification of phosphorylated PtrAldOMT by densitometric analysis. P-PtrAldOMT2 represent the phosphorylated PtrAldOMT2.

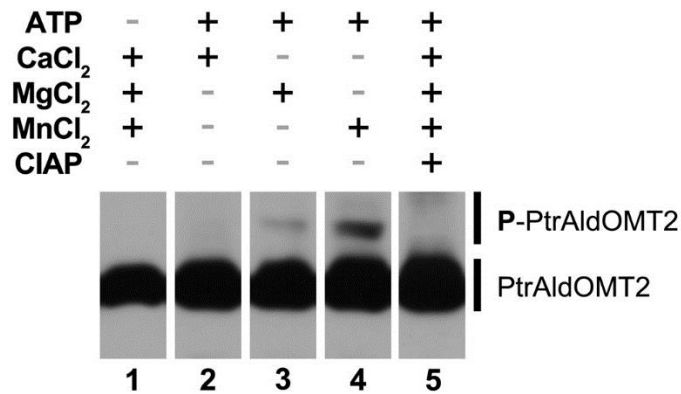


Figure 3-3 Recombinant PtrAldOMT2-6×His *in vitro* phosphorylation.

Lane 1: ATP is necessary for PtrAldOMT2 phosphorylation. Lane 2: Ca²⁺ cannot facilitate PtrAldOMT2 phosphorylation. Lane 3: Mg²⁺ weakly activates PtrAldOMT2 phosphorylation. Lane 4: Mn²⁺ effectively activates PtrAldOMT2 phosphorylation. Lane 5: CIAP dephosphorylates PtrAldOMT2.

3.3.2 Targeted LC-MS/MS Analysis Revealed *In vitro* Phosphorylated PtrAldOMT2 Recombinant Protein is Representative of Endogenous PtrAldOMT2

To further develop the phosphorylation system, and to confirm that the phosphorylation events introduced by the *in vitro* system are representative of the endogenous phosphorylation *in vivo*, and not due to nonspecific phosphorylation, the phosphorylation site location and occupancy of the *in vitro* phosphorylation system was next characterized. Recombinant PtrAldOMT2-6×His was incubated with SDX protein extracts with Mn^{2+} and ATP, and the reaction products were analyzed by high resolution MS/MS. PtrAldOMT2-6×His incubated in SDX protein extracts at 0°C was included as a negative control. Mono-phosphorylation at Ser¹²³ or Ser¹²⁵ was confirmed for recombinant PtrAldOMT2-6×His incubated for phosphorylation treatment, by the detection of phosphopeptides NEDGV(pS)VPLCLMNQDK and NEDGVSV(pS)PLCLMNQDK (**Fig. 3-4**). These peptides are identical to those phosphopeptides identified in previous global shotgun phosphoproteomics of *P. trichocarpa* SDX (**Fig. 2-1**). The relative retention time of the phosphorylated and unphosphorylated peptides, accurate intact mass (< 2 ppm) and fragment ion spectra were all consistent with global data and expected values. Consistent with the results of the global phosphoproteomics analysis was the lack of detectable PtrAldOMT2 derived peptides with phosphorylation at both Ser¹²³ and Ser¹²⁵ residues. The mono-phosphorylation is reasonable because the two phosphorylation sites are too close in proximity to allow for concurrent phosphorylation at both sites.

With the phosphorylation sites confirmed for the recombinant PtrAldOMT2, the levels of phosphorylation introduced by SDX protein extracts was next quantified. Total phosphorylation occupancy at Ser¹²³ and Ser¹²⁵ was increased 3.45 fold from $7.88 \pm 0.71\%$ in

the 0°C controls to $27.16 \pm 0.86\%$ by *in vitro* phosphorylation (**Fig. 3-5**). Phosphorylation observed for the controls is likely due to residual kinase activity at 0°C. This *in vitro* phosphorylation system can effectively increase the levels of phosphorylation of recombinant PtrAldOMT2, and the sites of phosphorylation induced are consistent with the endogenous phosphorylation sites in *P. trichocarpa* SDX. Using this *in vitro* phosphorylation system, purified phosphorylated recombinant PtrAldOMT2-6×His was then produced for further enzyme functional analysis.

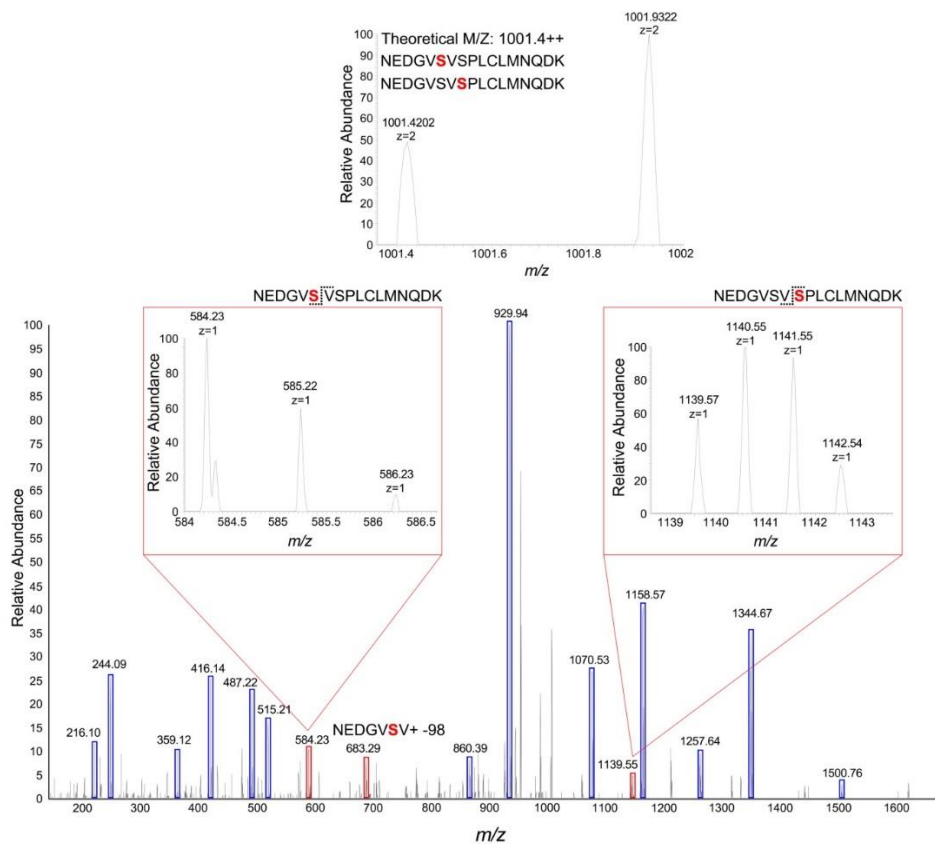


Figure 3-4 Targeted SIM data dependent MS/MS (TSIM-ddMS/MS) analysis of phosphorylated recombinant PtrAldOMT2 protein.

Red **S** represents the phosphorylated serine residues. Accurate MS1 within 2 ppm along with high sequence coverage by several MS/MS fragment ions within 0.02 Da allows for confident identification of phosphorylated peptides. Blue bars present fragment ions corresponding to both PtrAldOMT2 phosphopeptides. Red bars indicate specific fragment ions which uniquely identify S¹²³ and S¹²⁵ phosphorylation.

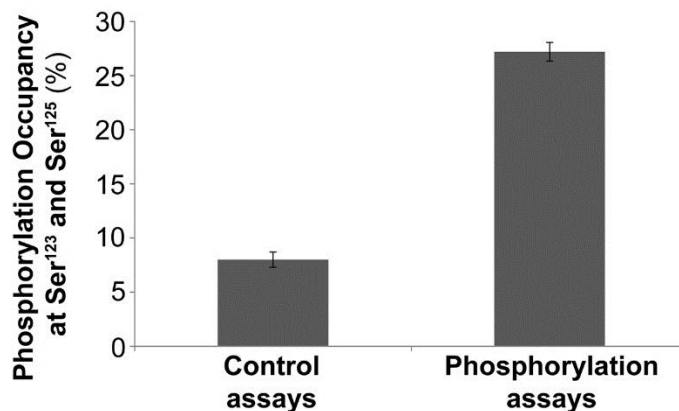


Figure 3-5 *P. trichocarpa* SDX protein extracts activated recombinant PtrAldOMT2-6×His phosphorylation.

Control assays: incubation at 0°C. Phosphorylation assays: incubation at 30°C. Error bars represent one standard error of five technical replicates.

3.3.3 The Ser¹²³ Phosphorylation Site is Highly Conserved in AldOMTs from Diverse Plant Species.

To elucidate the conservation of the Ser¹²³ and Ser¹²⁵ phosphorylation sites in plants, AldOMT protein sequences from 46 diverse vascular plant species were aligned (**Fig. 3-6**). AldOMTs in 45 of 46 (98%) plant species have either one or both PtrAldOMT2 phosphorylation sites (**Fig. 3-6**). *Medicago truncatula* is the species without either serine residues. The Ser¹²³ phosphorylation site is conserved across 43 of 46 (93%) AldOMTs (**Fig. 3-6**). The three plant species (*Gossypium hirsutum*, *Hibiscus cannabinus* and *Medicago truncatula*) without Ser¹²³ retained a phosphorylatable threonine or tyrosine residue in place of Ser¹²³. In contrast, the PtrAldOMT2 Ser¹²⁵ residue is only found in 8 of 46 (17%) AldOMTs, with small non-polar alanine or glycine residues in place of serine (**Fig. 3-6**).

Sequence alignment of 46 AldOMTs from diverse plant species showed phosphorylation sites that have been conserved or have diverged over evolutionary time. Given the presence of the Ser¹²³ phosphorylation site, this regulatory mechanism is likely to be of general occurrence

in plants. This suggests strong selection of AldOMT phosphorylation for regulation of monolignol biosynthesis. The presence of a second phosphorylatable serine (Ser¹²⁵) in AldOMT of *P. trichocarpa* and several other plant species suggests the emergence of redundancy for a vital function of phosphorylation.

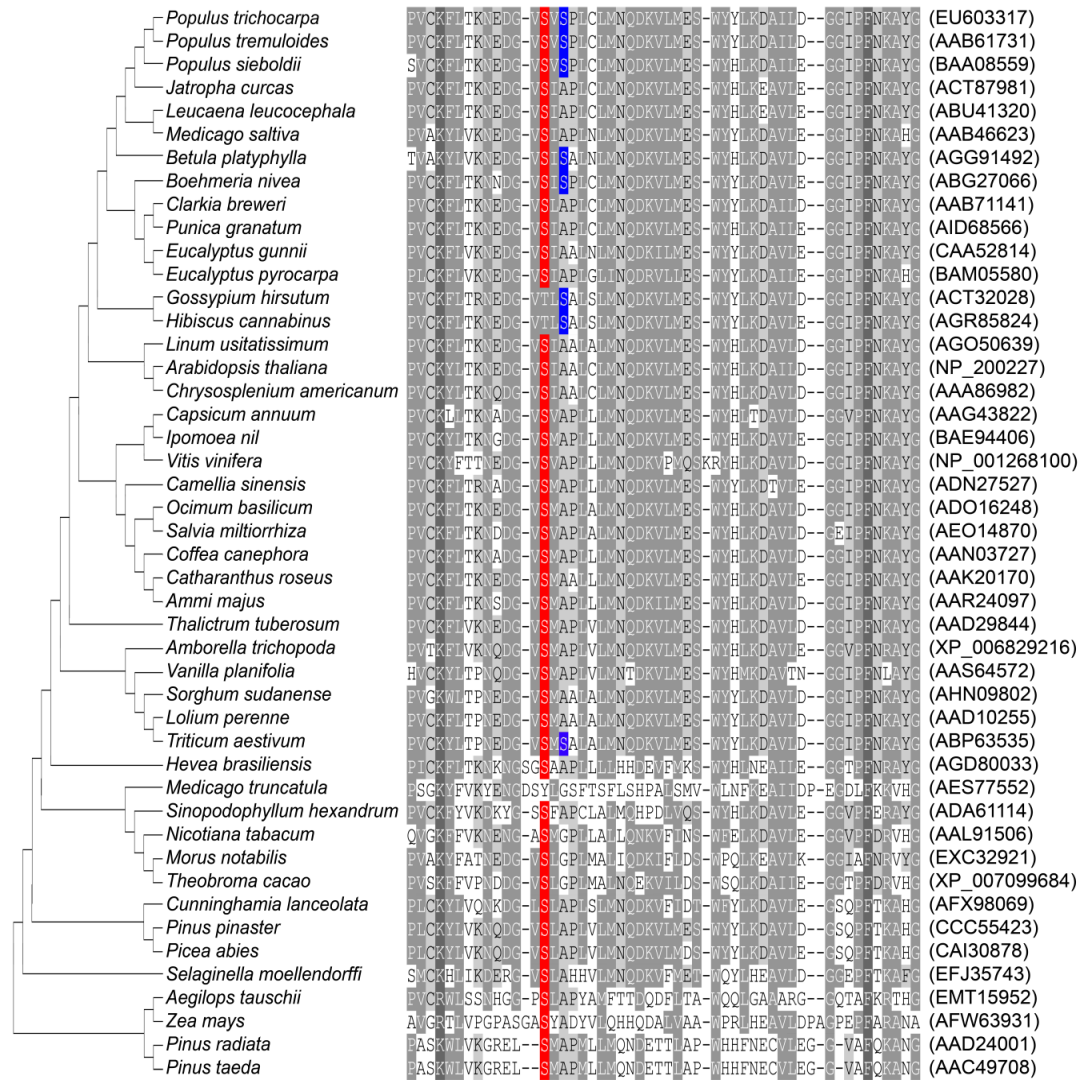


Figure 3-6 Neighbor joining phylogenetic tree and alignment of PtrAldOMT2 Ser¹²³ and Ser¹²⁵ residues to AldOMT from diverse plant species.

AldOMT protein sequences of 46 plant species were obtained from NCBI (www.ncbi.nlm.nih.gov) and aligned (110th to 160th residues of PtrAldOMT2 shown). Red highlights represent alignment to Ser¹²³ of PtrAldOMT2 and blue highlights represent alignment to Ser¹²⁵ of PtrAldOMT2. The GenBank accession numbers are listed in parenthesis.

3.4 Conclusion

The results of time-course analysis of the PtrAldOMT2-6XHis *in vitro* phosphorylation show that recombinant PtrAldOMT2-6XHis can be mono-phosphorylated *in vitro* and PtrAldOMT2-6XHis phosphorylation was most active when Mn²⁺ and ATP were added to the SDX protein extracts. The results of CIAP treatment of phosphorylated PtrAldOMT2-6XHis show the complete dephosphorylation of the recombinant protein can be readily achieved, suggesting plasticity in this post-translational modification. LC-MS/MS analysis of *in vitro* phosphorylated PtrAldOMT2-6XHis revealed the monophosphorylation on Ser¹²³ or Ser¹²⁵, which is consistent with the results of the global phosphoproteomics analysis was the lack of detectable PtrAldOMT2 derived peptides with phosphorylation at both Ser¹²³ and Ser¹²⁵ residues, suggesting that *in vitro* phosphorylated PtrAldOMT2 recombinant protein is representative of endogenous PtrAldOMT2. The levels of phosphorylation introduced by SDX protein extracts was quantified and show that total phosphorylation occupancy at Ser¹²³ and Ser¹²⁵ was increased 3.45 fold from 7.88 ± 0.71% in the 0°C controls to 27.16 ± 0.86% by *in vitro* phosphorylation. The highly conserved PtrAldOMT2 Ser¹²³ phosphorylation site in the results of the phylogenetic analysis of 46 AldOMT strongly suggests that such post-translational modification is an evolutionally important functional regulation of the *O*-methyltransferase in the monolignol biosynthetic pathway.

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Chapter 4 Functional Characterization of Phosphorylated 5-Hydroxyconiferaldehyde *O*-Methyltransferase

4.1 Introduction

5-hydroxyconiferaldehyde *O*-methyltransferase 2 (PtrAldOMT2) is an enzyme central to syringyl monolignol biosynthesis in *Populus trichocarpa*. AldOMT (EC 2.1.1.68) catalyzes the *S*-adenosyl-L-methionine (SAM) dependent methylation of 3- and 5- hydroxyl groups of precursors for syringyl monolignol biosynthesis (**Fig. 4-1**) (1). Due to the abundance of transcript and protein, regulation of PtrAldOMT2 activity by transcriptional control should be energy intensive and slow. Post-translational modifications may provide a mechanism for regulating PtrAldOMT2 activity without needing to synthesize/degrade protein; thus provides a rapid and energetically efficient mode of regulating *O*-methyltransferase activity for syringyl monolignol biosynthesis.

With the advent of phosphopeptide enrichment and high-throughput mass spectrometry, large scale global phosphoproteomic analysis has been applied to 20 plant species, and over 31,000 plant phosphoproteins have been identified (2). These data revealed phosphopeptides mapping to four of the ten monolignol biosynthetic enzyme families: one PAL of *Zea mays* and *Medicago truncatula*; one cinnamyl alcohol dehydrogenase (CAD) of *Arabidopsis thaliana*, *M. truncatula* and *Brachypodium distachyon*; and one cinnamoyl-CoA reductase (CCR) and one AldOMT of *A. thaliana* (2). Protein phosphorylation appears to be common in monolignol biosynthesis. However, no evidence has been presented practically to show functional regulation by protein phosphorylation for these monolignol pathway enzymes.

mutagenesis was performed using the QuikChange II Kit (Stratagene, La Jolla, CA) with the following primer sets: *OMT2-S123MF* and *OMT2-S123MR*, *OMT2-S125MF* and *OMT2-S125MR*, *OMT2-S123/125MF* and *OMT2-S123/125MR* (Table below). The resulting mutant plasmids were verified by DNA sequencing.

Table - Primers used for site-directed mutagenesis of *PtrAldOMT2*.

Primer Name	Sequence
OMT2-S123MF	GACAGAGAGGGCTGACATTGACACCATCCTCGTTCT
OMT2-S123MR	AGAACGAGGATGGTGTCAATGTCAGCCCTCTCTGTC
OMT2-S125MF	GACAGAGAGGGTTGACAGAGACACCATCCTCGT
OMT2-S125MR	ACGAGGATGGTGTCTCTGTCAACCCTCTCTGTC
OMT2-S123125MF	GTTTCATGAGACAGAGAGGGTTGACATTGACACCATCCTCGTTCTTG
OMT2-S123125MR	CAAGAACGAGGATGGTGTCAATGTCAACCCTCTCTGTCTCATGAAC

4.2.2 Enrichment of Phosphorylated Recombinant *PtrAldOMT2*

A phosphoprotein purification kit (QIAGEN, Venlo, Limburg) was used for enrichment of phosphorylated recombinant *PtrAldOMT2* without modification. After phosphorylation, the phosphorylated recombinant *PtrAldOMT2* was buffer exchanged using 5 mL lysis buffer, 1 tablet of protease inhibitor, 10 uL Benzonase Nuclease (DNase/RNase) provided by the QIAGEN kit with 100 uL of phosphatase inhibitor cocktail III (Sigma, St. Louis, MO). A 0.5 mL 30 kDa Amicon column (EMD Millipore, Millierica, MA) was used to desalt and concentrate the samples. The enrichment procedure was exactly as described in the QIAGEN kit. After enrichment, all the eluted fractions were buffer exchanged using repurification binding buffer (50 mM Tris-HCl pH 8.0, 400 mM NaCl, 10% v/v glycerol, phosphatase inhibitor (Sigma, St. Louis, MO), protease inhibitor (QIAGEN, Venlo, Limburg). For His-tagged recombinant protein purification, the desalted samples were resuspended in

repurification binding buffer and added onto a 2 mL Ni-NTA resin (Life technology, Carlsbad, CA) packed column. The protein bound resin was then washed with repurification washing buffer (50 mM Tris-HCl pH 8.0, 500 mM NaCl, 30 mM imidazole, 20 mM beta-mercaptoethanol, 10% v/v glycerol, phosphatase inhibitor (Sigma, St. Louis, MO)). The purified recombinant protein was eluted using repurification elution buffer (50 mM Tris-HCl pH 8.0, 500 mM NaCl, 250 mM imidazole pH 8.0, 20 mM beta-mercaptoethanol, 10% glycerol) then buffer was exchanged and the sample was concentrated in Tris-HCl pH 7.5, 20% v/v glycerol for protein storage and enzyme assays.

4.2.3 Enzyme Assays

The enzyme activity assays of phosphorylated and non-phosphorylated PtrAldOMT2 recombinant protein were performed using three substrates involved in monolignol biosynthesis (caffeic acid, caffealdehyde, and 5-hydroxyconiferaldehyde). Caffeic acid was purchased from Sigma Aldrich (St. Louis, MO). Caffealdehyde and 5-hydroxyconiferaldehyde were biochemically or chemically synthesized as described by Li et al. (3). Each substrate (final concentration of 100 μ M) was mixed with the assay solution containing buffer and cofactors (50 mM Tris-HCl pH 7.5, 2 mM MgCl₂, 1 mM SAM) to a final volume of 100 μ L (4). The reactions were performed in three replicates. The reaction mixtures were held at 30°C for 1 min followed by the addition of PtrAldOMT2 or SDX extract to initiate enzymatic reactions at 30°C for 30 min. The reactions were terminated by addition of 40 μ L of 5% trichloroacetic acid and 40% acetonitrile. The reaction mixture was then centrifuged at 20,000 \times g for 20 min to remove debris. 100 μ L of supernatant was used for HPLC analysis on an Agilent Zorbax SB-C3 5 μ m, 4.6 \times 150 mm column as described by Wang et al. (1).

4.3 Results and Discussion

4.3.1 Purified Phosphorylated Recombinant PtrAldOMT2 Has Essentially No *O*-Methyltransferase Activity

To determine how phosphorylation affects the *O*-methyltransferase function, purified phosphorylated recombinant PtrAldOMT2-6×His was produced, using the *in vitro* phosphorylation system described in a previous chapter and phosphate specific affinity chromatography. Protein fractions were analyzed by Phos-tag SDS-PAGE and western blotting using anti-His-antibody (**Fig. 4-2A**). *P. trichocarpa* SDX protein extracts phosphorylated 9% of the total recombinant protein (pre-enrichment, **Fig. 4-2A**). Using affinity chromatography, phosphorylation was increased to 45% of the total PtrAldOMT2-6×His (eluent, **Fig. 4-2A**). The absence of a protein band in the wash buffer (wash, **Fig. 4-2A**) indicates a complete separation of unphosphorylated PtrAldOMT2-6×His from their phosphorylated counterparts. The flow-through shows only a single band corresponding to the unphosphorylated PtrAldOMT2-6×His (flow-through, **Fig. 4-2A**), indicating successful retention of all phosphorylated PtrAldOMT2-6×His by affinity chromatography. The activity of the purified phosphorylated PtrAldOMT2-6×His was then assayed using caffeic acid, caffealdehyde or 5-hydroxyconiferaldehyde as the substrate.

When phosphorylated, the rate of conversion of caffealdehyde to coniferaldehyde was reduced to 4% of the unphosphorylated PtrAldOMT2-6×His control (**Fig. 4-2B**). No activity was detected for the phosphorylated PtrAldOMT2-6×His when either caffeic acid or 5-hydroxyconiferaldehyde was the substrate (**Fig. 4-2B**). These results showed that modification by phosphorylation inhibits PtrAldOMT2 enzyme activity. To confirm the phosphorylation

mediated inhibition of PtrAldOMT2 *in vivo*, how *O*-methyltransferase activity in *P. trichocarpa* SDX protein extracts is affected by phosphorylation was next investigated.

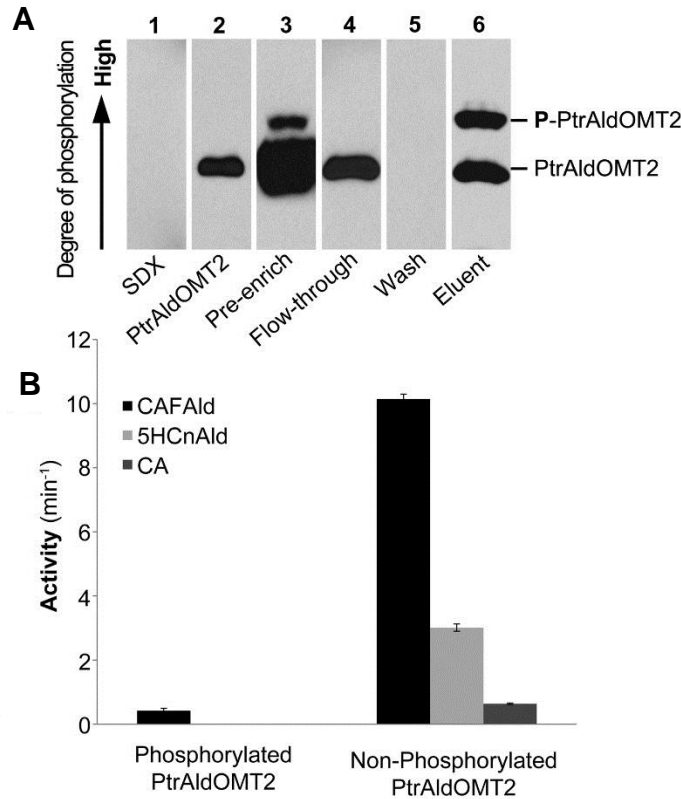


Figure 4-2 *O*-Methyltransferase activity assay of purified phosphorylated recombinant PtrAldOMT2.

(A) Phos-tag SDS-PAGE of phosho-enrichment of recombinant PtrAldOMT2 phosphorylated by *P. trichocarpa* SDX, immunodetected using anti-His antibody. Lane 1: SDX only control. Lane 2: PtrAldOMT2-6×His only control. Lane 3: Pre-enriched sample containing PtrAldOMT2-6×His and SDX. Lane 4: Flow-through. Lane 5: Wash buffer. Lane 6: Enriched sample containing phosphorylated PtrAldOMT2-6×His and phosphorylated SDX proteins. (B) Activity of unphosphorylated and phosphorylated PtrAldOMT2-6×His, with caffeic acid (CA), caffaealdehyde (CAFAld), or 5-hydroxyconiferaldehyde (5HCnAld) as substrate. Error bars represent one standard error of three technical replicates.

4.3.2 Phosphorylation Inhibits Endogenous PtrAldOMT2 Activity in *P. trichocarpa* SDX Protein Extracts

To confirm the phosphorylation-induced inhibition of PtrAldOMT2 enzyme activity, total protein extracts of *P. trichocarpa* SDX were treated with ATP and Mn^{2+} to promote phosphorylation of the endogenous SDX proteins (**Fig. 4-3**). Unphosphorylated native SDX protein extracts were included as a control. When phosphorylated, AldOMT activity in SDX was reduced to 44% of the control for conversion of caffeic acid to ferulic acid (**Fig. 4-3**), and to 49% of the control for conversion of caffealdehyde to coniferaldehyde (**Fig. 4-3**). The reduced AldOMT activity in SDX was not due to protein degradation, as the abundance of endogenous PtrAldOMT2 in SDX protein extracts was not reduced by phosphorylation (**Fig. 2-3**). CIAP treatment to remove phosphorylation restored AldOMT activity to the levels of the native SDX control (**Fig. 4-3**). PAL activity changes in the SDX protein extracts was also assayed. Phosphorylation and CIAP treatments did not alter the PAL activity in SDX compared to the control (**Fig. 4-3**), consistent with the observation that recombinant PAL catalytic efficiency (V_{max}/K_m) was unaffected by phosphorylation (5).

Suppression of enzyme activity by protein phosphorylation represents an important regulatory process in the complex control of plant metabolism (6). Key metabolic pathways such as sucrose and nitrogen metabolism are regulated by phosphorylation mediated enzyme suppression. For example: reversible phosphorylation of Ser¹⁵⁸ converts sucrose phosphate synthase (SPS) to a low-activity-form (7, 8); similarly, the phosphorylated form of nitrite reductase is inactive, whereas the dephosphorylated form is active (35). Reversible phosphorylation may also activate plant metabolic enzyme activities. Phosphorylation of Ser¹⁵ *in vitro* activates sucrose synthase (SuSy) activity, by increasing substrate affinities (36).

Although protein phosphorylation/dephosphorylation-dependent regulation is essential and ubiquitous in plant metabolism, the involvement of this post-translational modification in regulating monolignol biosynthesis had remained largely undefined. Having confirmed the phosphorylation mediated inhibition of PtrAldOMT2 activity, the functional roles of the individual PtrAldOMT2 phosphorylation sites was next investigated.

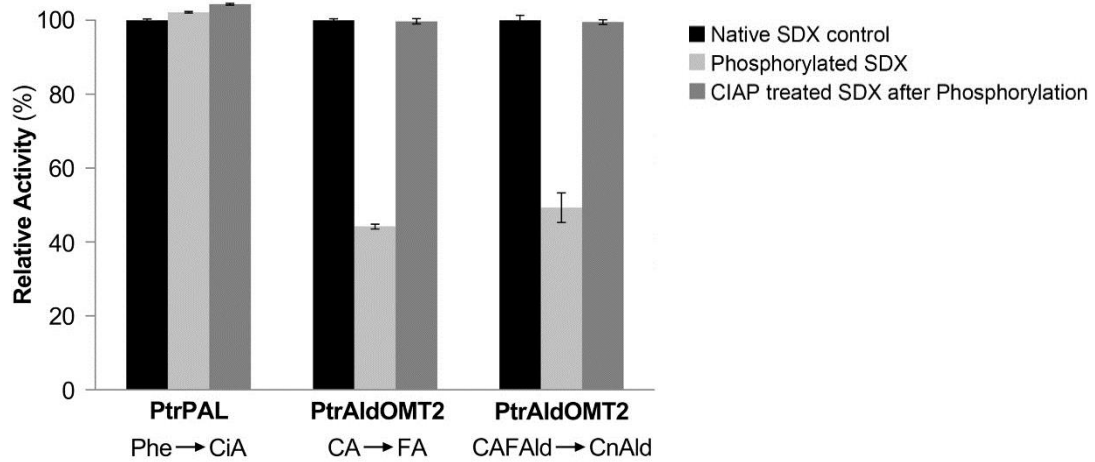


Figure 4-3 AldOMT and PAL activity in phosphorylated, CIAP treated and untreated native *P. trichocarpa* SDX protein extracts.

Abbreviations: phenylalanine (Phe); cinnamic acid (CiA); caffeic acid (CA); ferulic acid (FA); caffealdehyde (CAFAld); coniferaldehyde (CnAld). Error bars represent one standard error of three technical replicates.

4.3.3 Site-Directed Mutagenesis at Ser¹²³ and Ser¹²⁵ Verified PtrAldOMT2 Phosphorylation Sites and Their Functional Significance.

To further verify the identity of the Ser¹²³ and Ser¹²⁵ phosphorylation sites in PtrAldOMT2, and to directly assess the functional roles played by these serine residues, site-directed mutagenesis was used to convert Ser¹²³ and Ser¹²⁵ either singly or doubly to asparagine to produce S123N, S125N and S123N:S125N recombinant PtrAldOMT2-6×His. Asparagine is a non-phosphorylatable amino-acid that mimics the polar uncharged serine in the

unphosphorylated PtrAldOMT2 (9). Recombinant proteins of S123N, S125N and S123N:S125N were produced in *Escherichia coli*, purified to near homogeneity (**Fig. 4-4A**), and analyzed for phosphorylation by SDX protein extracts. As expected, mono-phosphorylation was observed when S123N or S125N was assayed for phosphorylation (lanes 3 and 4, **Fig. 4-4B**). Mutation of either Ser¹²³ or Ser¹²⁵ does not prevent phosphorylation of the other serine residue; the two phosphorylation sites are functionally independent. No phosphorylation was observed when the double mutant S123N:S125N was incubated in SDX protein extracts with Mn²⁺ and ATP (lane 5, **Fig. 4-4B**). This result confirmed that the phosphorylation of PtrAldOMT2 is exclusively limited to Ser¹²³ and Ser¹²⁵ residues, and that their mutation prohibited phosphorylation of PtrAldOMT2 by *P. trichocarpa* SDX protein extracts (lane 5, **Fig. 4-4B**). We next assessed their biochemical functions.

The *O*-methyltransferase activities of recombinant S123N, S125N and S123N:S125N was then analyzed, using caffeic acid, caffealdehyde or 5-hydroxyconiferaldehyde as substrate. When Ser¹²³ was converted to asparagine, *O*-methyltransferase activity was reduced to 6.9% of the unmodified PtrAldOMT2 control for caffeic acid (**Fig. 4-4C**), to 15.2% of the control for caffealdehyde (**Fig. 4-4C**), and to 9.5% of the control for 5-hydroxyconiferaldehyde (**Fig. 4-4C**). Inhibition of *O*-methyltransferase activity was less severe when Ser¹²⁵ was converted to asparagine. S125N activity was 35.9% of control for caffeic acid (**Fig. 4-4C**), 39.8% of control for caffealdehyde and 26.2% of control for 5-hydroxyconiferaldehyde (**Fig. 4-4C**). When both Ser¹²³ and Ser¹²⁵ were converted to asparagine, as in the case of S123N:S125N, *O*-methyltransferase activity for all three substrates was below the level of detection (**Fig. 4-4C**). The significant reduction in *O*-methyltransferase activity when Ser¹²³ and/or Ser¹²⁵ was modified confirmed that these serine residues are important for maintaining PtrAldOMT2

function, and that modifications to either one or both of these residues will drastically affect *O*-methyltransferase activity. In addition, the more severe inhibition observed for a modification of Ser¹²³ compared to Ser¹²⁵ suggest that the Ser¹²³ phosphorylation site plays a more effective regulatory role for the phosphorylation mediated control of PtrAldOMT2 activity. The redundant functions of Ser¹²³ and Ser¹²⁵ are suggested by the evidence that either site can undergo phosphorylation independently (**Fig. 4-4C**) and the significantly lower conservation of Ser¹²⁵ than Ser¹²³ (**Fig. 3-6**). Ser¹²³ and Ser¹²⁵ may also act as independent docking sites for distinct kinases and signaling pathways. Defining the temporal regulation of PtrAldOMT2 phosphorylation should yield new insights into the regulation of monolignol biosynthesis. Using site directed mutagenesis, the phosphorylation sites Ser¹²³ and Ser¹²⁵ were further confirmed as important sites for PtrAldOMT2 enzyme activity. The present study demonstrates that protein phosphorylation acts as an on/off regulatory switch for PtrAldOMT2 activity in monolignol biosynthesis of *P. trichocarpa*, providing an additional level of regulation for this important pathway. It was revealed that PtrAldOMT2 is mono-phosphorylated at Ser¹²³ or Ser¹²⁵ residues in wildtype SDX of *P. trichocarpa*. Moreover, the mono-phosphorylation of recombinant PtrAldOMT2 could be activated by incubation with *P. trichocarpa* SDX extracts containing endogenous kinases (**Fig. 3-2**). An important regulatory function of protein phosphorylation is the modulation (activation or suppression) of enzyme activity. Phosphorylation of PtrAldOMT2 results in the loss-of-function of the *O*-methyltransferase activity for monolignol biosynthesis. AldOMT activity is essential for syringyl monolignol biosynthesis (10), and on-off PtrAldOMT2 activity may be an efficient way to regulate syringyl monolignol metabolic-flux in the pathway (12).

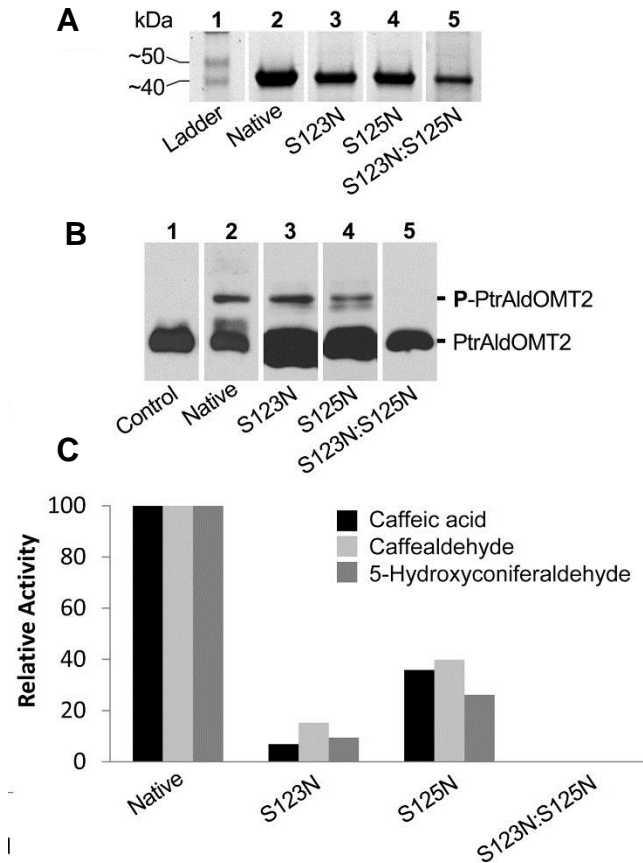


Figure 4-4 Site-directed mutagenesis of PtrAldOMT2 phosphorylation sites.

(A) Phos-tag SDS-PAGE of unphosphorylated PtrAldOMT2-6xHis site-directed mutagenesis at Ser¹²³ (S123N), Ser¹²⁵ (S125N), and both Ser¹²³ and Ser¹²⁵ (S123N:S125N). (B) Phos-tag SDS-PAGE of site-directed mutagenesis of PtrAldOMT2-6xHis immunodetected by anti-His antibody. Lane 1: PtrAldOMT2-6xHis only control. Lane 2: PtrAldOMT2-6xHis in SDX. Lane 3: S123N in SDX. Lane 4: S125N in SDX. Lane 5: S123N:S125N in SDX. (C) O-methyltransferase activity of native PtrAldOMT2-6xHis, S123N, S125N and S123N:S125N with caffeic acid, caffealdehyde or 5-hydroxyconiferaldehyde as substrates. Activities are single measurement determinations.

4.5 Conclusion

Phosphorylation turned off the PtrAldOMT2 activity, as demonstrated *in vitro* using purified phosphorylated and unphosphorylated recombinant PtrAldOMT2. Protein extracts of *P. trichocarpa* SDX, which contains endogenous kinases, also phosphorylated recombinant PtrAldOMT2 and turned off the recombinant protein activity. Similarly, ATP/Mn²⁺-activated

phosphorylation of SDX protein extracts reduced the endogenous SDX PtrAldOMT2 activity by approximately 60%, and dephosphorylation fully restored the activity. Phosphorylation-site mutagenesis verified the PtrAldOMT2 phosphorylation at Ser¹²³ or Ser¹²⁵, and confirmed the functional importance of these phosphorylation sites for *O*-methyltransferase activity. The reversible phosphorylation of PtrAldOMT2 is likely to have an important role in regulating syringyl monolignol biosynthesis of *P. trichocarpa*.

To meet environmental and developmental needs, the monolignol biosynthetic pathway for lignification in plant cell walls is regulated by complex mechanisms involving transcriptional, post-transcriptional, and metabolic controls. However, post-translational modification by protein phosphorylation had not been demonstrated in the regulation of monolignol biosynthesis. Here, it was revealed that reversible mono-phosphorylation at Ser123 or Ser125 acts as an on/off switch for the activity of PtrAldOMT2. Phosphorylation induces a loss-of-function of PtrAldOMT2, which directly affects metabolic-flux for syringyl monolignol biosynthesis. Protein phosphorylation provides a rapid and energetically efficient mode of regulating PtrAldOMT2 activity for syringyl monolignol biosynthesis, and represents an additional level of control for this important pathway.

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