Girdhar K. Pandey *Editor*

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Functional Genomic Perspective

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Editor
Girdhar K. Pandey
Department of Plant Molecular Biology
University of Delhi South Campus
New Delhi, India

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Preface

The famous Sun temple of Konark in India represents the chariot of the Sun God (Surya) that is pulled by seven horses. This beautiful structure carved out of stone is a good example of management where the seven horses must be controlled for the proper movement of the chariot. The charioteer controls the movement of the chariot through a "leash" that is tied to each of the horses. Nature too has its own set of controls that act as leashes to modulate the nuances of cellular functions. This book is dedicated to the understanding of one such group of controls in plants, which function as highly effective components of the signal transduction mechanism.

In today's scenario, human health, food security, natural resources like water, air, and soil (pollution as well as conservation) are the pertinent issues affecting mankind. Though food security is a major challenge at all levels, it is particularly important for the plant biologists, especially in the context of climate change and scarcity of arable land. Environmental stresses (both abiotic and biotic) and extensive anthropogenic activities have contributed significantly to the drastic decline in crop productivity over the years. Researchers have made significant agronomic advances by crop improvement through extensive breeding and to some extent by genetic manipulation. The genomic and post-genomic eras have witnessed the use of advanced tools of gene manipulation and genetic engineering, to target a large number of genes for imparting stress tolerance in plants. Plant biologists are trying to understand and explore the structural and functional relationship of gene(s) and gene families, in the context of different physiological and developmental aspects of the plant life cycle. However, the efforts to develop stress-tolerant crop varieties have not been much successful. In lieu of this, we still require a detailed understanding of the mechanisms underlying stress perception, transduction as molecular signals, and finally translation into defense or adaptive responses by plants.

Signaling pathways act as "nodes and hubs," regulating myriad stimuli including stress signals. These nodes and hubs also regulate the cross talk and disparate channeling of stress signals and hence fine-tune the stimulus–response-coupling process with the generation of adaptive responses. These mechanisms maintain a homeostatic balance in the living systems in concomitance with stress perception and the ensuing responses. There are several controls or molecular switches which turn

these biological processes and signaling pathways "on and off." Posttranslational modifications of a protein can act as one of the key molecular switches in an organism. Protein phosphorylation is one such covalent modification that regulates signaling cascades through the activation or deactivation of the components involved in maintenance of a homeostatic state. This process of reversible regulation is carried out through two groups of enzymes, kinases, and phosphatases. Kinases phosphorylate target proteins by adding a phosphate to the hydroxyl group on amino acid residues, while phosphatases dephosphorylate a phosphorylated protein, thereby forming a cellular switch to initiate or terminate diverse cellular processes. Protein phosphosites mainly include nine amino acids: tyrosine (Tyr), serine (Ser), threonine (Thr), cysteine (Cys), arginine (Arg), lysine (Lys), aspartate (Asp), glutamate (Glu), and histidine (His). Ser-, Thr-, and Tyr- are the most commonly phosphorylated residues with profound implications in the regulatory pathways in eukaryotic cells.

Protein kinases (PK) are known to be activated by primary stress response pathways (such as in Ca^{2+} signaling) which subsequently elicit either a short-term quick response like closing and opening of the stomata or a long-term response like activation of transcription factors. However, if this response is not switched off and persists, the plant would be allocating its resources toward adaptive responses even in the absence of stress. Thus, the resource allocation of the plant will tip more toward protecting itself rather than a holistic growth response even after the stress has been mitigated. This aspect of the regulation of stress response pathways dictates the need for a discussion on protein phosphatases (PPs). These PPs are important components which control many regulatory circuits in living organisms by modulating the conformation, activity, localization, and stability of substrate proteins. PPs are categorized depending on their biochemical properties. The three main families of PP are Ser-/Thr-, Asp-, and Tyr-based. The Ser/Thr-based PPs are divided into the phosphoprotein phosphatase (PPP) family and the metallo-dependent PPs (PPM/PP2C) family. The Asp-based PPs are divided into the FCP-like/CPL and HAD families. The Tyr-based PPs are further divided into the protein Tyr phosphatases (PTPs) and dual specificity phosphatases (DsPTPs).

The phosphorylation–dephosphorylation-regulated "cellular switch" that monitors plant physiology, growth, and development has immense potential in crop systems. Much of the information pertaining to this regulatory mechanism in plants is still in the nascent stages, coming largely from model plants, Arabidopsis and rice. The use of genetic and biochemical approaches aided by "omic" approaches are currently enabling the unraveling of key components involved in the regulation of stress tolerance. These key components of phosphorylation–dephosphorylation such as kinases or phosphatases could be exploited to develop crop varieties better equipped to handle adverse environmental conditions and hence lead to enhancement of agricultural productivity.

This book entitled *Protein phosphatases and stress management in plants: Functional genomic perspective* comprises of 17 chapters contributed by several well-known plant biologists working in the field of Protein phosphatases and stress management with a special emphasis on Functional Genomic aspect. This book

elaborates on the state-of-art scientific advances in the field of "signaling under stress conditions," which will formulate a holistic understanding on the subject.

The first chapter describes the role of ancient chloroplast and mitochondrial PPs, the Shewanella-like PPs (SLP1 and SLP2), of bacterial origin. They are remarkably conserved in plants, suggesting that they play fundamental roles in chloroplast and mitochondrial biology. The detailed functional role of SLP1 and SLP2 is being investigated in several plant species, especially in the physiological and functional context.

Chapters 2 and 3 elaborate the role of the purple acid phosphatases (PAPs). The PAPs are involved in phosphate (Pi) homoeostasis and several other diverse functions such as regulation of seed traits, root development, osmotic, oxidative and salt stress tolerance in plants. Chapter 4 discusses the PP2A class of protein phosphatases that are composed of three subunits (catalytic "C"; scaffolding "A" and regulatory "R"). The Arabidopsis genome encodes multiple isoforms of these subunits (3-As, 5-Cs, and 17-Bs subunits), and different combinations of these subunits are expected to give rise to almost 255 different PP2A holoenzymes. Though PP2A are mostly implicated in the regulation of developmental pathways, but in this chapter, authors discuss their possible role in responses to salinity stress.

PP2Cs belonging to PPM family are the largest class of PPs in plants. Arabidopsis and rice encode more than 76 and 90 PP2Cs, respectively, that are classified into 10 or more subgroups (A–K) with diverse functions. The best studied PPs include the A-subclade of PP2C, which negatively regulate the ABA signaling pathway. Chapters 5 and 6 discuss the role of PP2Cs in regulating diverse abiotic stresses and ABA signaling. Chapter 7 presents a detailed insight into the role of PP2A and PP2C families in sugar as well as hormone signaling and consequently in the maintenance of balance between stress and growth in plants.

The opening and closing of the stomata are governed by guard cell dynamics which control their turgid state. Guard cell signaling is one of the most well-studied physiological processes in plants, wherein an intricate interplay of PKs and PPs is at work. Chapter 8 gives a detailed account of several PPs such as PP1s, PP2As, and PP2Cs in the regulation of the stomatal movements. Chapter 9 presents an insight into the involvement of several PPs in the regulation of plant responses under salt stress in different species. Chapter 10 discusses the role of phosphatases and different phosphatase gene families involved in stress signaling pathways, involved in the regulation of stress tolerance.

Because crop productivity is directly dependent on the soil fertility and nutrient content, mineral nutrient deficiency in plants is an important area which demands greater attention from plant biologists. Deficiency of the major- and micro-nutrients in the soil leads to a drastic penalty in growth and development, thus affecting the crop yield and productivity. A large number of fertilizers are added to different crops to enhance the yield and productivity. Among the fertilizers, NPK (nitrogen, phosphorus, and potassium) is the most commonly preferred combination. Chapter 11 presents the role of various PPs in the regulation of responses to the K^+ deficiency and the signaling therein. Moreover, this chapter also discusses the importance of Ca^{2+}-mediated CBL-CIPK (a homologue of the animal PP2B class

phosphatase calcineurin) and PP2C modules in the regulation of K^+ transport, ABA and abiotic stress signaling pathways in the model plant Arabidopsis. Chapter 12 elaborates the role of PPs in nitrogen response and nitrogen-use efficiency (NUE) in different crops. Several PPs such as PP2Cs, PP2As, and others, identified in different N uptake, assimilation, and remobilization regulatory pathways, are emerging as important candidate genes for genetic manipulation. Chapter 13 elaborates on the genome-wide identification of PPs from major cereals and small grain crops, their structural organization as well as their involvement in diverse stress regulatory pathways. This chapter lays emphasis on PPs from the perspective of crop plants.

Besides regulating the cytoskeletal network comprising of microtubule and actin filaments, PPs act as important determinants of cell cycle progression and thus regulators of cell division. Chapter 14 presents the roles of various PPs in mitotic processes and cytoskeleton regulation. In addition to abiotic stresses, biotic challenges posed by pests and pathogens affect crop productivity drastically. To cope with biotic stresses, plants have different layers of defense systems such as pattern-triggered immunity (PTI) and effector triggered immunity (ETI). However, parallelly, forces of natural selection aid pathogens in evolving a more effective arsenal of defense mechanisms. Successful invasion by pathogens and the consequent defense responses in plants solely depends on the host–pathogen interactions, which comprise of a large number of components that trigger several signaling pathways. Chapter 15 provides an extensive account of involvement of PPs in host–pathogen interactions in both host and pathogen systems.

In animals, both Ser/Thr and Tyr phosphorylation–dephosphorylation regulate a large number of physiological and developmental processes. However, till date, no receptor tyrosine kinase (RTK) has been identified in plants, though several reports suggest Tyr phosphorylation by non-canonical Tyr kinases. Tyr dephosphorylation by Tyr-specific phosphatases is also not much explored. Based on the genome sequence analysis of several plant species, not many PTPs have been identified. Chapters 16 and 17 present an account of dual specificity phosphatases (DSPs; which act on both phosphorylated Ser/Thr and Tyr) and Tyr-specific phosphatases (PTP) in different plants. Their involvement in the regulation of different metabolic (starch degradation), physiological (biotic and abiotic stresses), and various developmental processes is discussed.

Plants need to acquire a large number of reprograming in their biological processes that enable them to withstand the changing nature of their environment. Based on the extensive work done in the field of stress perception and signal transduction, it is evident that research in the area of signal transduction is a key determinant in the implementation of enhanced stress tolerance in plants. My best efforts were rendered toward the inclusion of all aspects of PPs and their role in stress management in this book. However, some aspects still await elaboration due to space constraint and other limitations. Regardless of this, I firmly believe that this book will be able to serve its purpose for students, researchers, and academicians seeking an understanding of stress-mediated signaling in the context of PPs.

I express my gratitude to all the authors whose contributions have made it possible to bring vast information on one platform. I also express my sincere thanks to

Dr. Malathi Bheri, Dr. Sibaji K. Sanyal, and Dr. Deepti M. Nambiar (Department of Plant Molecular Biology, University of Delhi South Campus, New Delhi, India) for their critical and constructive suggestions. I am also thankful to Delhi University, University Grant Commission, Department of Biotechnology, Science and Engineering Research Board, and Council of Scientific and Industrial Research, India, for supporting the research in my laboratory.

New Delhi, India Girdhar K. Pandey

Contents

Author's Biography

Girdhar K. Pandey received his B.Sc. (Hon.) in Biochemistry from Delhi University in 1992 and M.Sc. in Biotechnology in the year 1994 from Banaras Hindu University (BHU). Subsequently, he joined PhD in the School of Life Sciences, Jawaharlal Nehru University (JNU), and worked in the field of calcium signal transduction under abiotic stresses in plants. He was awarded the PhD degree in the year 1999 and then pursued postdoctoral career at Department of Plant and Microbial Biology, University of California at Berkeley in the year 2000. There, he extended his work in the field of calcium-mediated signaling in Arabidopsis by studying CBL-CIPKs, phosphatases, channels/transporters, and transcription factors involved in abiotic stresses. Currently, he is working as Professor in the Department of Plant Molecular Biology, Delhi University South Campus.

Dr. Pandey's research interests involve detail mechanistic interplay of signal transduction networks in plants under mineral nutrient deficiency (mostly potassium, calcium, and nitrate) and abiotic stresses such as drought, salinity, and oxidative stresses induced by heavy metals. His laboratory is working on the coding and decoding of mineral nutrient deficiency and abiotic stress signals by studying several signaling components such as calcium sensors such as calcineurin B-like (CBL) and CBL-interacting protein kinases (CIPK), phosphatases (mainly PP2C and DSP), transcription factors (AP2-domain containing or ERF, WRKY), transporters and channels proteins (potassium and calcium channels/transporters) in both Arabidopsis and rice. The long-term goal of his research group is to establish the mechanistic interplay and cross talk of mineral nutrient-deficient conditions and different abiotic stress signaling cascades in Arabidopsis and rice model system by using the advance tools of bioinformatics, genetics, cell biology, biochemistry, and physiology with greater emphasis on functional genomics approaches.

See Dr. Pandey's web page for further information about his lab and research work: https://sites.google.com/site/gkplab/home; http://www.dpmb.ac.in/index.php?page=girdhar-pandey

Chapter 1
SLP1 and SLP2: Ancient Chloroplast and Mitochondrial Protein Phosphatases

Jayde J. Johnson, Chris White-Gloria, Ryan Toth, Anne-Marie Labandera, R. Glen Uhrig, and Greg B. Moorhead

1.1 Introduction

The covalent modification of proteins is now regarded as a common post-translational mechanism to regulate protein function in all organisms. Phosphorylation was the first protein covalent modification to be discovered and has its origins in the history of glycogen metabolism and signal transduction research (Brautigan and Shenolikar 2018). Up to ten different amino acids that occur in proteins can be phosphorylated, with serine, threonine, and tyrosine being the most common. Although varying slightly across organisms and cellular conditions, a typical phospho-proteome is about 86% phospho-serine, 12% phospho-threonine, and 2% phospho-tyrosine (Sharma et al. 2014; van Wijk et al. 2014; White-Gloria et al. 2018). The recent development of monoclonal antibodies that specifically recognize phospho-histidine has uncovered roles for this modification in eukaryotes (Adam and Hunter 2018). The development of mass spectrometry technologies related to phosphoproteomics, especially quantitative mass spectrometry, has established protein phosphorylation as the most common covalent modification in all organisms explored, including a variety of plant species (Sharma et al. 2014; van Wijk et al. 2014; White-Gloria et al. 2018).

Protein phosphorylation is not just a cytosolic and nuclear phenomenon; new mass spectrometry data have also established protein phosphorylation as a common event in chloroplasts (White-Gloria et al. 2018; Baginsky and Gruissem 2009;

J. J. Johnson · C. White-Gloria · R. Toth · G. B. Moorhead (✉)
Department of Biological Sciences, University of Calgary, Calgary, AB, Canada
e-mail: moorhead@ucalgary.ca

A.-M. Labandera
Plant Proteolysis and Signaling Laboratory, School of Biosciences, University of Birmingham, Birmingham, UK

R. G. Uhrig
Department of Biological Sciences, University of Alberta, Edmonton, AB, Canada

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Reiland et al. 2009; Richter et al. 2016; Pagliarini and Dixon 2006), mitochondria (Baginsky and Gruissem 2009; Pagliarini and Dixon 2006; Bykova et al. 2003; Grimsrud et al. 2012), and peroxisomes (Oeljeklaus et al. 2016; Kataya et al. 2019). Uncovering the abundant cache of protein phosphorylation events in eukaryotes is no surprise given the enormous size of the protein kinase and phosphatase gene families. For instance, human and Arabidopsis genomes encode ~518 and ~942 protein kinases and ~189 and ~150 protein phosphatase catalytic subunits, respectively (Chen et al. 2017; Kerk et al. 2008; Uhrig et al. 2013a). Unlike kinases, the number of protein phosphatases is in fact less than the above-quoted figures as several of these do not act on protein substrates but are included in this group based on sequence. Many protein phosphatases, predominantly the PPP family enzymes, have additional regulatory subunits that dictate their function. The association of a variety of unrelated regulatory subunits with a common catalytic subunit balances the apparent disparity in protein kinase and phosphatase numbers. This multitude of regulatory subunits also brings specificity to what are regarded as somewhat promiscuous catalytic subunits (Brautigan and Shenolikar 2018; Moorhead et al. 2007, 2008, 2009; Bollen et al. 2010; Heroes et al. 2013; Nasa et al. 2018).

1.2 Protein Phosphatases in Eukaryotes

The protein phosphatases in eukaryotes belong to four separate families known as PPP (phosphoprotein phosphatases), PPM/PP2C (Mg^{2+}-dependent protein phosphatases), Asp-based protein phosphatases, and PTP (phospho-tyrosine phosphatases). The majority of phospho-serine and phospho-threonine dephosphorylation is catalyzed by PPP and PPM family members. PPP members include PP1, PP2 (PP2A), PP3 (PP2B), and PP4–7. It is notable that plants do not possess PP3 (PP2B) class of phosphatases but instead are endowed with additional novel members such as ALPH, RLPH, SLP1, and SLP2 (Pagliarini and Dixon 2006; Kerk et al. 2008; Uhrig et al. 2013b). Here, we provide an update on the SLP1 and SLP2 enzymes that function in chloroplasts and mitochondria, respectively. As mentioned above, it is the variety of additional subunits that bind the PPP catalytic subunits and bring specificity to the enzymes, and we predict this is also true for the SLPs.

1.3 Chloroplast and Mitochondrial Protein Phosphorylation

It is now well accepted that protein phosphorylation is the most common covalent modification of proteins in eukaryotes, with a majority of phosphoproteins residing in the cytosol and nucleus. Less is known about the phospho-proteome of mitochondria and chloroplasts, although phosphoproteins were identified in these organelles in 1969 (in animals) (Linn et al. 1969; Miernyk and Randall 1987) and 1977 (Miernyk and Randall 1987; Bennett 1977), respectively. In fact, the first *in vitro*

demonstration of protein kinase activity in 1954 was the phosphorylation of casein by a mitochondrial extract (Pagliarini and Dixon 2006; Burnett and Kennedy 1954). The pyruvate dehydrogenase complex (PDC) is composed of three components with the E1 subunit being the first mitochondrial phosphoprotein to be discovered in animals, followed soon by the plant E1 subunit (Miernyk and Randall 1987). PDC catalyzes the reaction that yields acetyl-CoA and NADH from pyruvate and NAD^+. PDC activity is regulated by the inactivating phosphorylation by pyruvate dehydrogenase kinase (PDK) and the activating dephosphorylation by the PP2C-like enzyme, a phospho-pyruvate dehydrogenase phosphatase (PDP). Multiple proteins in the mitochondrial matrix and intermembrane space have now been identified as phosphoproteins. In 2013, 64 phosphorylated proteins and 10 protein kinases were identified in plant mitochondria (Havelund et al. 2013). More recently, Law et al. (2018) found that out of 802 mitochondrial proteins, 103 were found to have experimentally determined phosphorylation sites in just the first 60 N-terminal amino acids with implications in mitochondrial targeting (Law et al. 2018).

Recent chloroplast specific and general phospho-proteomic studies have illustrated widespread protein phosphorylation in the chloroplast (Baginsky and Gruissem 2009; Reiland et al. 2009; Richter et al. 2016). This is consistent with studies identifying multiple protein kinases and phosphatases in this organelle (Baginsky and Gruissem 2009; Richter et al. 2016; Andreeva and Kutuzov 2004), including casein kinase 2α4 (CK2α4), STN7, STN8, three thylakoid-associated kinases (TAKs), chloroplast sensor kinase (CSK), a family of atypical protein kinases (Activity of BC1 Complex Kinase or ABC1K), several Plastid Protein Kinases With Unknown Function (PKUs), seven type-2C phosphatases (PP2C), TAP38, PBCP, and SLP1 (White-Gloria et al. 2018; Uhrig and Moorhead 2011). We recently reviewed phosphorylation of the chloroplast starch metabolic machinery and cataloged phosphorylation of most of these enzymes (White-Gloria et al. 2018). Note that the plastid enzymes SEX4, LSF1, and LSF2 are designated phosphatases based on sequence, yet they are not protein phosphatases, acting as either scaffolds or starch phosphatases (Silver et al. 2014).

Clearly, protein phosphorylation is a regulatory mechanism that has been well established in mitochondria and chloroplasts. The explosion of phospho-proteomic data has revealed greater phosphorylation in these organelles than originally anticipated. This observation is simply consistent with a flood of phospho-proteomic data revealing abundant protein phosphorylation in bacteria, the ancient origin of these organelles.

1.4 Discovery and Bioinformatics of SLP1 and SLP2

Shewanella-like protein phosphatases 1 and 2, or SLP1 and SLP2, were identified in a bioinformatic study and given the name *Shewanella*-like protein phosphatase based on their sequence relationship to a PPP-like serine/threonine phosphatase of this bacterium (Andreeva and Kutuzov 2004). This bacterial origin is consistent

with the mitochondrial and chloroplast localization in eukaryotes as these two organelles have been hypothesized to have had their origins in symbiotic bacteria. All key residues that define the PPP family phosphatases (GDxHG, GDxVDRG, GNHE, and HGG (Shi 2009)) are present in the SLPs, suggesting they are true protein phosphatases (Fig. 1.1) (Andreeva and Kutuzov 2004; Uhrig and Moorhead 2011). Orthologues of SLP1 and SLP2 are present in organisms across four of the five major eukaryotic supergroups (plantae, opisthokonts, chromalveolates, and excavates). The SLPs predominate in photosynthetic organisms with no SLP genes in animals (Uhrig et al. 2013b). All plant species examined contain SLP1 and SLP2 genes. In addition, we uncovered a third, more ancient group of SLP phosphatases (SLP3 phosphatases) in green algae (Uhrig et al. 2013b). Arabidopsis *thaliana* SLP2 is an intronless protein phosphatase, and exploring SLP2 across higher plants shows an almost complete absence of introns in the SLP2 gene (Uhrig and Moorhead 2017). The lack of introns is consistent with many mitochondrial destined proteins (Uhrig and Moorhead 2017). Key features, including the chloroplast transit peptide (cTP) on SLP1 orthologues, are shown in the alignment displayed in Fig. 1.1.

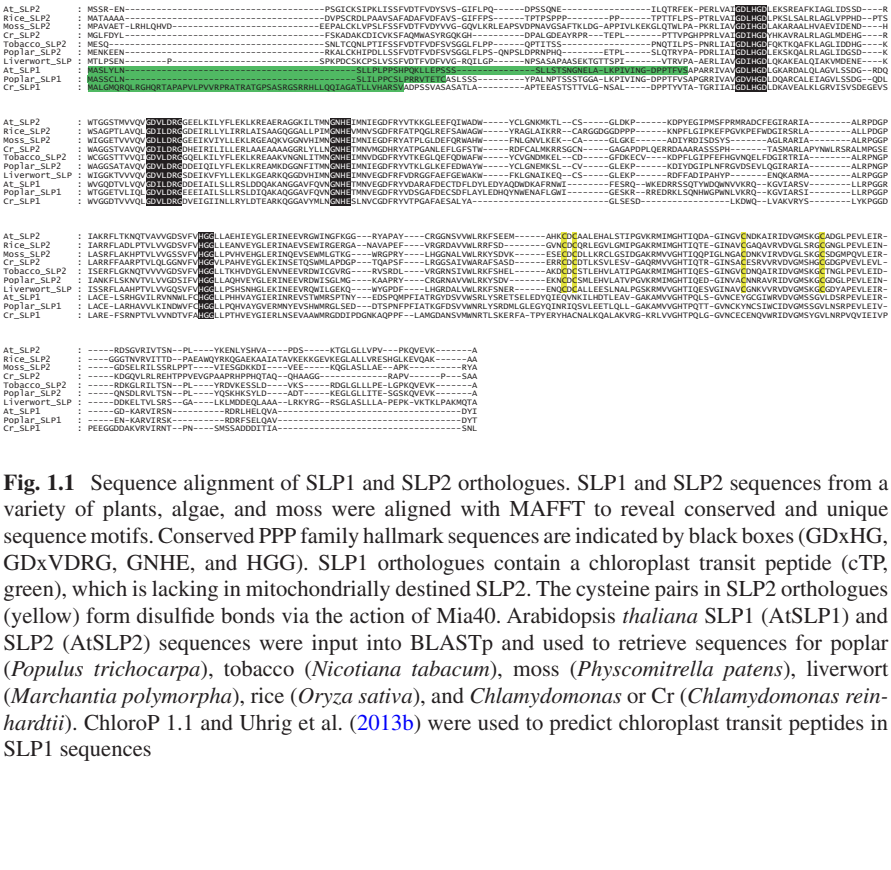

Fig. 1.1 Sequence alignment of SLP1 and SLP2 orthologues. SLP1 and SLP2 sequences from a variety of plants, algae, and moss were aligned with MAFFT to reveal conserved and unique sequence motifs. Conserved PPP family hallmark sequences are indicated by black boxes (GDxHG, GDxVDRG, GNHE, and HGG). SLP1 orthologues contain a chloroplast transit peptide (cTP, green), which is lacking in mitochondrially destined SLP2. The cysteine pairs in SLP2 orthologues (yellow) form disulfide bonds via the action of Mia40. Arabidopsis *thaliana* SLP1 (AtSLP1) and SLP2 (AtSLP2) sequences were input into BLASTp and used to retrieve sequences for poplar (*Populus trichocarpa*), tobacco (*Nicotiana tabacum*), moss (*Physcomitrella patens*), liverwort (*Marchantia polymorpha*), rice (*Oryza sativa*), and *Chlamydomonas* or Cr (*Chlamydomonas reinhardtii*). ChloroP 1.1 and Uhrig et al. (2013b) were used to predict chloroplast transit peptides in SLP1 sequences

1.5 SLP1 Is a Chloroplast-Localized Serine/Threonine Protein Phosphatase

Using fluorescent protein tagged version of AtSLP1 and multiple markers for cellular compartments, AtSLP1 was demonstrated to be chloroplast localized, consistent with bioinformatics that predicted a chloroplast transit peptide in AtSLP1 and most other SLP1 orthologues (Uhrig et al. 2013b; Uhrig and Moorhead 2011) (Fig. 1.1). This is consistent with western blotting of various tissues revealing that AtSLP1 is only expressed in photosynthetic tissues. Biochemically, AtSLP1 is insensitive to the classic PPP protein phosphatase inhibitors okadaic acid and microcystin but is remarkably sensitive to inorganic phosphate (Pi) and pyrophosphate (PPi). Although AtSLP1 has the hallmarks of a PPP family serine/threonine phosphatase, it displays activity against serine, threonine, and tyrosine phosphorylated peptides (Uhrig and Moorhead 2011). Current evidence suggests that no tyrosine phosphorylation occurs in the chloroplast (White-Gloria et al. 2018). AtSLP1 activity against tyrosine phosphorylated peptides may reflect the fact that it resides in the chloroplast and may not need to maintain stringent or specific serine/threonine phosphatase activity. This activity against phospho-tyrosine was displayed by both the bacterial expressed protein and TAP-AtSLP1 produced *in planta* (Uhrig et al. 2016). To date, no SLP1 substrates or regulatory subunits have been identified.

1.6 SLP2 Is a Mitochondrial Intermembrane Space Serine/Threonine Protein Phosphatase

Although the closest relative of SLP1 is SLP2, sequence differences are readily apparent. Arabidopsis *thaliana* SLP2 (AtSLP2) does not have a chloroplast transit peptide (cTP) but does have a series of cysteines critical to AtSLP2 function (Fig. 1.1). We have biochemically characterized AtSLP2 and used TAP (tandem affinity purification)-tag coupled to mass spectrometry to identify binding partners. Using this approach, a single clear binding partner for SLP2 has emerged: the oxidoreductase Mia40. Reverse TAP (TAP-Mia40) and co-immunoprecipitation confirmed Mia40 binding to SLP2 (Uhrig et al. 2017). Mia40 has been characterized in yeast and human cells as a mitochondrial intermembrane space protein that mediates the formation of disulfide bonds on target proteins. It has been demonstrated that both Mia40 and SLP2 reside in the mitochondrial intermembrane space, while some population of Mia40 also localizes to peroxisomes (Uhrig et al. 2017). Although a direct interactor of AtSLP2, Mia40 only modifies the enzyme and is not regarded as a regulatory subunit. Like AtSLP1, no regulatory subunits have been identified for AtSLP2, and it is also insensitive to the inhibitors okadaic acid and microcystin. Also, similar to SLP1, bacterially produced AtSLP2 displays activity against serine, threonine, and tyrosine phosphorylated peptides, but the *in planta* made TAP-AtSLP2 showed a remarkable preference for phospho-threonine over phospho-tyrosine using the same peptide substrates (Uhrig et al. 2017).

An insertional knockout of AtSLP2 (atslp2-2) was screened for growth phenotypes, and an accelerated germination phenotype was uncovered (Uhrig et al. 2017). This could be reversed when the knockout line was complemented with AtSLP2 driven by the endogenous promoter. Overexpression of AtSLP2 delays germination. In a knockout of Mia40 (*atmia40*), seeds exhibited a moderate accelerated germination phenotype consistent with SLP2 being the driver of the phenotype and likely displaying partial activity *in vivo* in the absence of Mia40 (Uhrig and Moorhead 2011).

1.7 Mia40 as a Redox Regulator

Specific mechanisms for the translocation of proteins through the outer mitochondrial membrane using the translocase of outer membrane (TOM) complex and then into specific mitochondrial sub-compartments have been known for some time (Fig. 1.2). However, it was not until 2004 that a protein was discovered in *Saccharomyces cerevisiae* which specifically targeted proteins to the mitochondrial intermembrane space (IMS), such as the small TIM proteins (Chacinska et al. 2004). They termed this protein as mitochondrial intermembrane space import and assembly protein 40, or Mia40. Experimental evidence showed that small TIM proteins

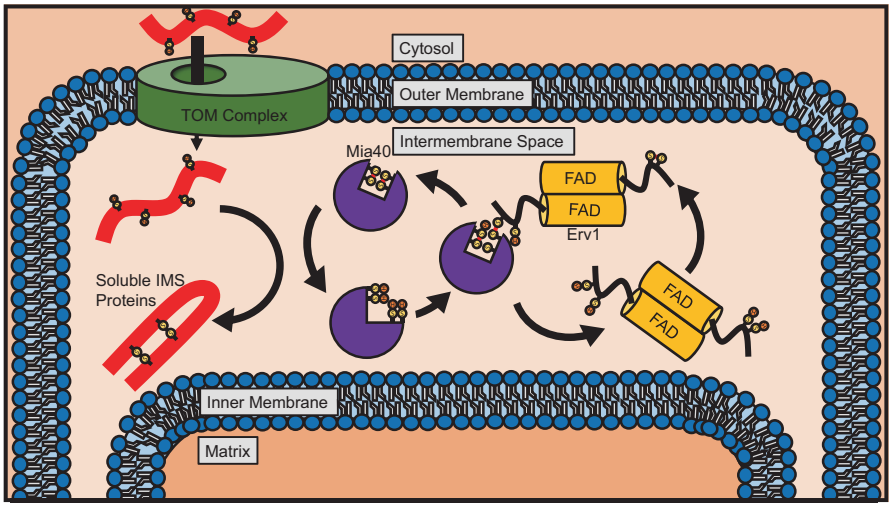

Fig. 1.2 Oxidoreductase Mia40 activates target proteins, including SLP2, in the mitochondrial intermembrane space. Proteins destined to reside in the mitochondrial intermembrane space (IMS) enter through the TOM complex and upon association with oxidized Mia40 form intramolecular disulfide bonds via pairs of conserved cysteines (see Fig. 1.1). IMS destined proteins (red) enter with reduced cysteines that are targeted by Mia40. By accepting electrons from target proteins, Mia40 is reduced and must be reoxidized by Erv1. In yeast, it has been demonstrated that Erv1 is reoxidized by transferring electrons to cytochrome c; this step has yet to be formally shown in plants

are imported into the IMS by TOM in a partly folded conformation and Mia40 then aids in the proper folding of proteins in the IMS. The mechanism of Mia40 was elucidated in 2005 (Mesecke et al. 2005) when it was found that proteins imported through TOM contained conserved cysteine motifs necessary for their import. A disulfide relay system was proposed in which Mia40 and another protein, Erv1, a sulfhydryl oxidase, compose the disulfide relay (Fig. 1.2). In this relay, Erv1 oxidizes cysteine residues in Mia40 allowing it to modify cysteines of imported proteins. Through disulfide bond isomerization, the proteins are then folded in the IMS to their fully functional, native structures. Yeast cells which lack Erv1 and therefore harbor Mia40 protein with reduced cysteines in their mitochondria lack viability due to the inability to successfully import and fold mitochondrial IMS proteins.

Mia40 activates the phosphatase activity of recombinant AtSLP2 ~4-fold when the artificial substrate pNPP is used, and this is dependent upon reductant, consistent with Mia40 generating disulfides on the enzyme to activate it. Although Mia40 does not alter the substrate specificity of AtSLP2 (i.e., pSer, pThr versus pTyr), it increases activity against substrate peptides up to 35-fold. Importantly, we demonstrated that Mia40 has no effect on AtSLP1 (Uhrig et al. 2017). To date, no AtSLP2 substrates have been identified.

1.8 MS-Based Substrate Discovery: The Future of Protein Phosphatases?

The discovery of protein phosphatase substrates has been technically challenging and always lags behind advances in protein kinase substrate discovery. Advances in mass spectrometry methods, in particular quantitative mass spectrometry, have changed the scenario now (Nasa et al. 2018; Rusin et al. 2015). It is now possible to knock out specific protein phosphatases and through quantitative analysis identify phosphopeptides that increase in the absence of the phosphatase, making the proteins these phospho-peptides are derived from as putative direct substrates. Although there are limitations in this approach, it is expected to revolutionize protein phosphatase substrate elucidation, and we expect this will be a common approach for protein phosphatase studies in the near future.

1.9 Conclusions

Bioinformatic, cell biological, and biochemical studies have established SLP1 and SLP2 as protein phosphatases that reside in the chloroplast and mitochondrial intermembrane space, respectively. Sequence analysis of SLP1 and SLP2 shows they are "bare" catalytic subunits with no accessory domains to regulate their activity. PPP family phosphatases typically associate with other proteins that regulate their

function in the cell. To date, no regulatory subunits for either SLP1 or SLP2 have been identified. Understanding the functions and roles for each enzyme will require identifying these regulatory subunits and finding their substrates. Only then can we assign clear biological functions for these proteins. We speculate that this will be aided by quantitative mass spectrometry.

References

Adam K, Hunter T (2018) Histidine kinases and the missing phosphoproteome from prokaryotes to eukaryotes. Lab Investig 98:233–247

Andreeva AV, Kutuzov MA (2004) Widespread presence of "bacterial-like" PPP phosphatases in eukaryotes. BMC Evol Biol 4:47

Baginsky S, Gruissem W (2009) The chloroplast kinase network: new insights from large-scale phosphoproteome profiling. Mol Plant 2:1141–1153

Bennett J (1977) Phosphorylation of chloroplast membrane polypeptides. Nature 269:344–346

Bollen M, Peti W, Ragusa MJ, Beullens M (2010) The extended PP1 toolkit: designed to create specificity. Trends Biochem Sci 35:450–458

Brautigan DL, Shenolikar S (2018) Protein serine/threonine phosphatases: keys to unlocking regulators and substrates. Annu Rev Biochem 87:921–964

Burnett G, Kennedy EP (1954) The enzymatic phosphorylation of proteins. J Biol Chem 211:969–980

Bykova NV, Egsgaard H, Moller IM (2003) Identification of 14 new phosphoproteins involved in important plant mitochondrial processes. FEBS Lett 540:141–146

Chacinska A, Pfannschmidt S, Wiedemann N, Kozjak V, Sanjuan Szklarz LK, Schulze-Specking A, Truscott KN, Guiard B, Meisinger C, Pfanner N (2004) Essential role of Mia40 in import and assembly of mitochondrial intermembrane space proteins. EMBO J 23:3735–3746

Chen MJ, Dixon JE, Manning G (2017) Genomics and evolution of protein phosphatases. Sci Signal 10:eaag1796

Grimsrud PA, Carson JJ, Hebert AS, Hubler SL, Niemi NM, Bailey DJ, Jochem A, Stapleton DS, Keller MP, Westphall MS, Yandell BS, Attie AD, Coon JJ, Pagliarini DJ (2012) A quantitative map of the liver mitochondrial phosphoproteome reveals posttranslational control of ketogenesis. Cell Metab 16:672–683

Havelund JF, Thelen JJ, Moller IM (2013) Biochemistry, proteomics, and phosphoproteomics of plant mitochondria from non-photosynthetic cells. Front Plant Sci 4:51

Heroes E, Lesage B, Gornemann J, Beullens M, Van Meervelt L, Bollen M (2013) The PP1 binding code: a molecular-lego strategy that governs specificity. FEBS J 280:584–595

Kataya AR, Muench DG, Moorhead G (2019) A framework to investigate peroxisomal protein phosphorylation in arabidopsis. Trends Plant Sci 24:366–381

Kerk D, Templeton G, Moorhead GB (2008) Evolutionary radiation pattern of novel protein phosphatases revealed by analysis of protein data from the completely sequenced genomes of humans, green algae, and higher plants. Plant Physiol 146:351–367

Law YS, Ngan L, Yan J, Kwok LY, Sun Y, Cheng S, Schwenkert S, Lim BL (2018) Multiple kinases can phosphorylate the N-terminal sequences of mitochondrial proteins in Arabidopsis thaliana. Front Plant Sci 9:982

Linn TC, Pettit FH, Reed LJ (1969) Alpha-keto acid dehydrogenase complexes. X. Regulation of the activity of the pyruvate dehydrogenase complex from beef kidney mitochondria by phosphorylation and dephosphorylation. Proc Natl Acad Sci U S A 62:234–241

Mesecke N, Terziyska N, Kozany C, Baumann F, Neupert W, Hell K, Herrmann JM (2005) A disulfide relay system in the intermembrane space of mitochondria that mediates protein import. Cell 121:1059–1069

Miernyk JA, Randall DD (1987) Some properties of pea mitochondrial phospho-pyruvate dehydrogenase-phosphatase. Plant Physiol 83:311–315
Moorhead GB, Trinkle-Mulcahy L, Ulke-Lemee A (2007) Emerging roles of nuclear protein phosphatases. Nat Rev Mol Cell Biol 8:234–244
Moorhead GB, Trinkle-Mulcahy L, Nimick M, De Wever V, Campbell DG, Gourlay R, Lam YW, Lamond AI (2008) Displacement affinity chromatography of protein phosphatase one (PP1) complexes. BMC Biochem 9:28
Moorhead GB, De Wever V, Templeton G, Kerk D (2009) Evolution of protein phosphatases in plants and animals. Biochem J 417:401–409
Nasa I, Rusin SF, Kettenbach AN, Moorhead GB (2018) Aurora B opposes PP1 function in mitosis by phosphorylating the conserved PP1-binding RVxF motif in PP1 regulatory proteins. Sci Signal 11:eaai8669
Oeljeklaus S, Schummer A, Mastalski T, Platta HW, Warscheid B (2016) Regulation of peroxisome dynamics by phosphorylation. Biochim Biophys Acta 1863:1027–1037
Pagliarini DJ, Dixon JE (2006) Mitochondrial modulation: reversible phosphorylation takes center stage? Trends Biochem Sci 31:26–34
Reiland S, Messerli G, Baerenfaller K, Gerrits B, Endler A, Grossmann J, Gruissem W, Baginsky S (2009) Large-scale Arabidopsis phosphoproteome profiling reveals novel chloroplast kinase substrates and phosphorylation networks. Plant Physiol 150:889–903
Richter AS, Gartmann H, Fechler M, Rodiger A, Baginsky S, Grimm B (2016) Identification of four plastid-localized protein kinases. FEBS Lett 590:1749–1756
Rusin SF, Schlosser KA, Adamo ME, Kettenbach AN (2015) Quantitative phosphoproteomics reveals new roles for the protein phosphatase PP6 in mitotic cells. Sci Signal 8:rs12
Sharma K, D'Souza RC, Tyanova S, Schaab C, Wisniewski JR, Cox J, Mann M (2014) Ultradeep human phosphoproteome reveals a distinct regulatory nature of Tyr and Ser/Thr-based signaling. Cell Rep 8:1583–1594
Shi Y (2009) Serine/threonine phosphatases: mechanism through structure. Cell 139:468–484
Silver DM, Kotting O, Moorhead GB (2014) Phosphoglucan phosphatase function sheds light on starch degradation. Trends Plant Sci 19:471–478
Uhrig RG, Moorhead GB (2011) Two ancient bacterial-like PPP family phosphatases from Arabidopsis are highly conserved plant proteins that possess unique properties. Plant Physiol 157:1778–1792
Uhrig RG, Moorhead G (2017) AtSLP2 is an intronless protein phosphatase that co-expresses with intronless mitochondrial pentatricopeptide repeat (PPR) and tetratricopeptide (TPR) protein encoding genes. Plant Signal Behav 12:e1307493
Uhrig RG, Labandera AM, Moorhead GB (2013a) Arabidopsis PPP family of serine/threonine protein phosphatases: many targets but few engines. Trends Plant Sci 18:505–513
Uhrig RG, Kerk D, Moorhead GB (2013b) Evolution of bacteria-like phosphoprotein phosphatases in photosynthetic eukaryotes features ancestral mitochondrial or archaeal origin and possible lateral gene transfer. Plant Physiol 163:1829–1843
Uhrig RG, Labandera AM, Muhammad J, Samuel M, Moorhead GB (2016) Rhizobiales-like phosphatase 2 from Arabidopsis thaliana is a novel phospho-tyrosine-specific phospho-protein phosphatase (PPP) family protein phosphatase. J Biol Chem 291:5926–5934
Uhrig RG, Labandera AM, Tang LY, Sieben NA, Goudreault M, Yeung E, Gingras AC, Samuel MA, Moorhead GB (2017) Activation of mitochondrial protein phosphatase SLP2 by MIA40 regulates seed germination. Plant Physiol 173:956–969
van Wijk KJ, Friso G, Walther D, Schulze WX (2014) Meta-analysis of Arabidopsis thaliana phospho-proteomics data reveals compartmentalization of phosphorylation motifs. Plant Cell 26:2367–2389
White-Gloria C, Johnson JJ, Marritt K, Kataya A, Vahab A, Moorhead GB (2018) Protein kinases and phosphatases of the plastid and their potential role in starch metabolism. Front Plant Sci 9:1032

Chapter 2
Phosphoprotein Phosphatase Function of Secreted Purple Acid Phosphatases

Mina Ghahremani and William C. Plaxton

Abbreviations

APase	Acid phosphatase
ECM	Extracellular matrix
ER	Endoplasmic reticulum
HAD	Haloacid dehalogenase
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
MS	Mass spectrometry
PAP	Purple acid phosphatase
Pi	Orthophosphate
P-Ser	Phosphoserine
PSI	Pi starvation-inducible
P-Thr	Phosphothreonine
PTM	Posttranslational modification
P-Tyr	Phosphotyrosine
VLK	Vertebrate lonesome kinase

2.1 Introduction

Acid phosphatases (APases; E.C. 3.1.3.2) catalyze the hydrolysis of orthophosphate (Pi, HPO_4^{2-}) from Pi monoesters and anhydrides with acidic pH optima. Purple APases (PAPs) represent the largest class of plant APases and exist as a diverse family of metallohydrolases involved in a multitude of biological processes. These include Pi-ester hydrolysis to facilitate plant Pi acquisition and the generation of

M. Ghahremani
Department of Biology, University of Ottawa, Ottawa, ON, Canada

Department of Biology, Queen's University, Kingston, ON, Canada

W. C. Plaxton (✉)
Department of Biology, Queen's University, Kingston, ON, Canada
e-mail: plaxton@queensu.ca

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reactive oxygen species as an immune response in mammals (Schenk et al. 2013; Tran et al. 2010a; Wang and Liu 2018). In contrast to other APases, PAPs are not inhibited by L(+)-tartrate. Hence, PAPs are also known as tartrate-resistant APases. Eukaryotic PAPs are glycoproteins that display highly variable amino acid sequences and sizes. However, their active sites are highly conserved, with seven invariant amino acid side chains coordinating Fe^{3+} and M^{2+} metal cations (M = Fe^{2+} in animals; Zn^{2+} or Mn^{2+} in plants) that participate in the catalytic mechanism for Pi-ester hydrolysis (Schenk et al. 2013). A charge-transfer transition from a conserved metal-coordinating tyrosine residue to the Fe^{3+} metal ligand leads to a typical absorption peak around 510–560 nm and a distinctive pink or purple color of PAPs in solution. Plant PAPs belong to a relatively large gene family encoding a diverse suite of isozymes. For example, the genome of the model plant Arabidopsis *thaliana* contains 29 *PAP* genes whose transcription is dependent upon various developmental and environmental factors (Li et al. 2002) (Fig. 2.1). These PAPs have been classified into two major groups according to their size, i.e., low molecular weight "mammalian-like" PAPs of approximately 35 to 45-kDa and higher molecular weight PAPs that range from about 50 to 70 kDa (Fig. 2.1). By contrast, only a single PAP isozyme of about 35-kDa (ACP5) has been characterized from animals. As discussed below, human ACP5 (HsACP5) is associated with microbial killing and bone resorption through its peroxidase and protein phosphatase activities, respectively (Schenk et al. 2013). Bioinformatics has identified a second *PAP-like* gene termed *ACP7* in mammals and other animal phyla that encodes a 55-kDa polypeptide that is more closely related to high molecular weight plant PAPs than it is to the low molecular weight ACP5 (Fig. 2.1) (Flanagan et al. 2006). Subsequent transcriptome profiling via RNA-seq indicated that human *ACP7* (*HsACP7*) is transcribed in various tissues, particularly skin and brain (https://www.ncbi.nlm.nih.gov/gene/390928). However, there have been no follow-up studies of the protein expression levels, biochemical properties, or function of the putative plant-like, high molecular weight ACP7 of humans or other animals (G. Schenk, personal communication).

Most PAPs that have been biochemically characterized are classified as nonspecific APases that catalyze Pi hydrolysis from a broad spectrum of Pi monoesters (Schenk et al. 2013; Tran et al. 2010a). This is consistent with their central role in cellular Pi metabolism, particularly scavenging and recycling Pi from intra- and extracellular Pi esters during nutritional Pi deprivation or senescence of vascular plants (Stigter and Plaxton 2015; Tran et al. 2010a; Wang and Liu 2018). However, HsACP5 expressed in macrophages also plays a role in immunity via the generation of reactive oxygen species (via a Fenton reaction involving the "redox-active" Fe^{2+} of their catalytic site) (Schenk et al. 2013). Similarly, several plant PAPs that function as APases also exhibit peroxidase activity that may contribute to the metabolism of reactive oxygen species during biotic or abiotic stress (Li et al. 2008; Tran et al. 2010a). The aim of this chapter is to briefly review the central role of PAPs in mediating plant Pi acquisition and use, followed by a discussion (1) of protein phosphorylation networks in the extracellular matrix (ECM) of animal and plant tissues

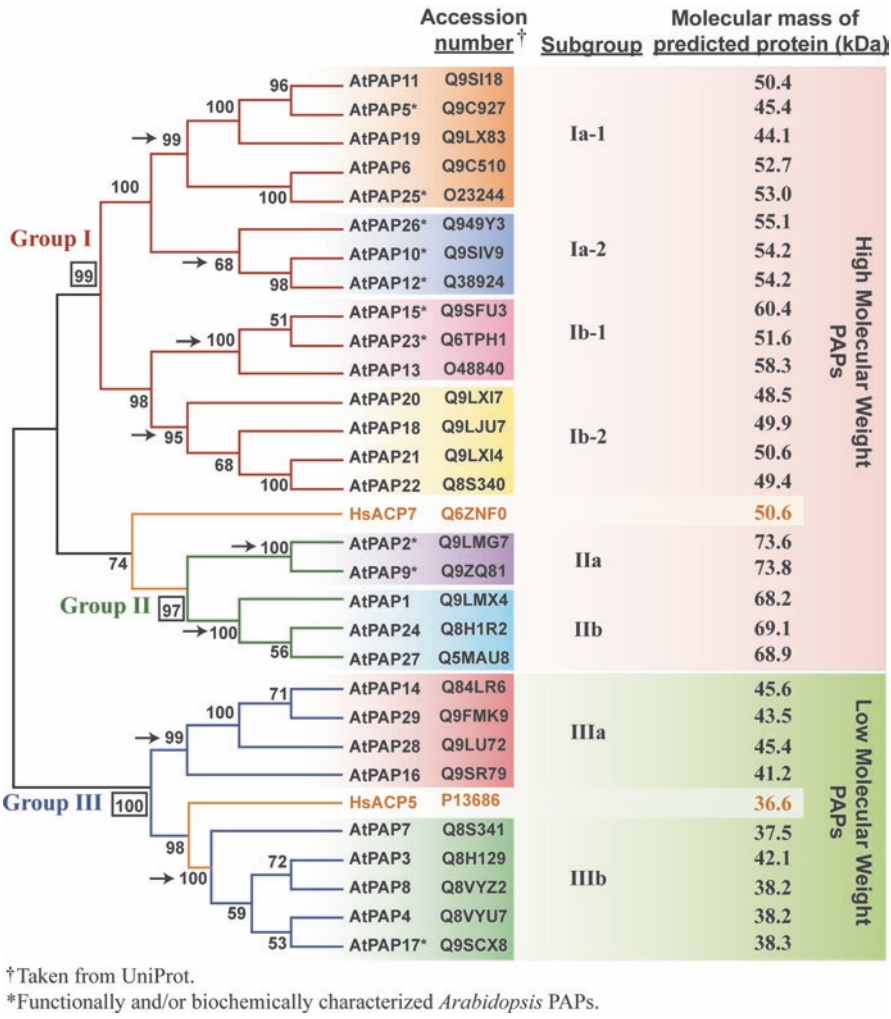

Fig. 2.1 Classification of Arabidopsis *thaliana* PAPs (AtPAPs) and *Homo sapiens* PAPs (HsACP5 and HsACP7) based on clustering analysis of amino acid sequence. For AtPAPs, the clustering analysis used amino acid sequences of 19 predicted PAPs and those of ten PAPs (AtPAP3, AtPAP7–AtPAP13, AtPAP17, and AtPAP18) derived from cDNA analysis. AtPAPs possess three main groups (groups I, II, and III), which are further divided into subgroups. The deduced amino acid sequences of two human PAPs, HsACP7 and HsACP5, were obtained from UniProt (https://www.uniprot.org) and aligned with AtPAPs in MUSCLE 3.8 using ClustalW. A maximum likelihood tree was constructed in MEGA 7.0 using WAG model with the gamma distributed with invariant sites (G + l) and the partial deletion options. The bootstrap values for the three main groups are boxed, and the bootstrap values for the subgroups are indicated by arrows. The predicted molecular masses of the deduced polypeptides are listed in the last column. (Figure modified from Li et al. 2002)

and (2) that certain PAP isozymes secreted by animal and plant cells appear to function as phosphoprotein phosphatases rather than as nonspecific scavengers of Pi from extracellular Pi monoesters.

2.1.1 PAPs Play a Central Role in Plant Pi Acquisition and Use Efficiency

Phosphorus is an essential element for growth and metabolism because it plays a central role in nearly all-metabolic processes. Roots preferentially absorb phosphorus from the soil in its fully oxidized anionic form, Pi (Fig. 2.2). Despite its importance, Pi is one of the least available macronutrients in many terrestrial and aquatic environments (Plaxton and Tran 2011; Tran et al. 2010a; Veneklaas et al. 2012). In soil, Pi frequently forms insoluble precipitates with metal cations such as Al^{3+} and Ca^{2+} or is converted into organic P molecules by soil microbes that therefore render it unavailable for direct root uptake (Fig. 2.2). Thus, plants needed to evolve adaptations that facilitate their acclimation to extended periods of nutritional Pi deficiency (within species-dependent limits) by eliciting a complex array of morphological, physiological, and biochemical adaptations, collectively known as the Pi-starvation response. The Pi-starvation response arises in part from the coordinated induction of hundreds of *Pi-starvation-inducible* (PSI) genes that reprioritize internal Pi use and maximize external Pi acquisition and includes (1) extending the root's surface area for Pi absorption, (2) root excretion of organic acid anions such as malate and citrate to mobilize Pi from insoluble Pi-metal cation complexes in the soil, and (3) the induction of high-affinity Pi transporters as well as alternative bypass enzymes to the Pi- or adenylate-dependent reactions of central metabolism (Fig. 2.3) (Plaxton and Tran 2011). Upregulation of certain vacuolar and secreted (cell wall and apoplast) PAP isozymes is another important aspect of plant Pi-starvation responses. Numerous studies have characterized PSI PAPs to define the molecular mechanisms underlying this archetypal response of Pi-deprived plants, as well as to identify potential targets for the biotechnological improvement of crop Pi acquisition and use efficiency (Tran et al. 2010a; Wang and Liu 2018). Transgenic PAP expression offers a promising approach for sustainable crop Pi nutrition since organic P typically constitutes at least 50% of the total P in soils and is the predominant form of P found in soil solutions (Fig. 2.2).

Integrated biochemical and functional genomic studies have identified the closely related AtPAP10, AtPAP12, and particularly AtPAP26 (Fig. 2.1) as the predominant PAP isozymes that are upregulated and secreted into the ECM by Pi-deprived Arabidopsis suspension cells and seedlings (Hurley et al. 2010; Robinson et al. 2012b; Tran et al. 2010b; Veljanovski et al. 2006; Wang et al. 2014). Their widespread and reversible upregulation in roots and shoots of Pi-starved Arabidopsis, overlapping but nonidentical substrate selectivities and pH-activity profiles, and high specific APase activities support the hypothesis that they collectively mediate

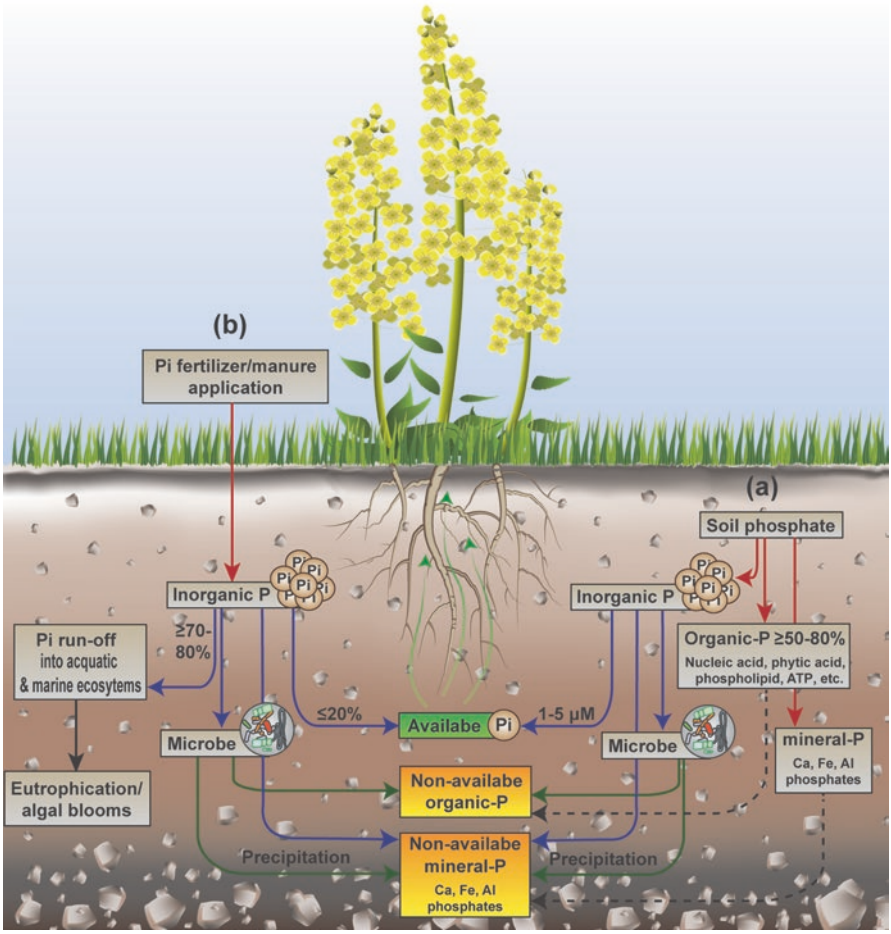

Fig. 2.2 Model of soil phosphorus (P) cycle. Most soils across the world are highly Pi limited since their soluble Pi concentration typically ranges between 1 and 5 μM, which is far below the intracellular Pi concentration (5–20 mM) required for optimal plant growth. (**a**) The lack of available Pi is due to several factors: (1) Pi is leached out of the soil due to the negative charge of clay, (2) Pi is converted into organic P forms by microorganisms, and (3) Pi can precipitate as insoluble calcium salts and iron or aluminum oxides in alkaline and acidic soils, respectively. (**b**) Less than 20% of Pi fertilizer applied worldwide per year is typically assimilated by crops. The remainder is either (1) converted into organic P by soil microbes; (2) bound by metal cations such as Al^{3+}, forming insoluble complexes; or (3) lost as runoff into nearby surface waters, resulting in the nutrient enrichment of aquatic and marine ecosystems and consequent "blooms" of green algae and toxic cyanobacteria; this stubborn environmental problem has caused extensive eutrophication

efficient Pi scavenging and recycling from a broad range of extracellular Pi esters (Fig. 2.3). Indeed, growth of wild-type Arabidopsis seedlings on media containing glycerol-3-phosphate (an effective *in vitro* substrate of AtPAP10, AtPAP12, and AtPAP26) as their sole source of exogenous P was indistinguishable from that of

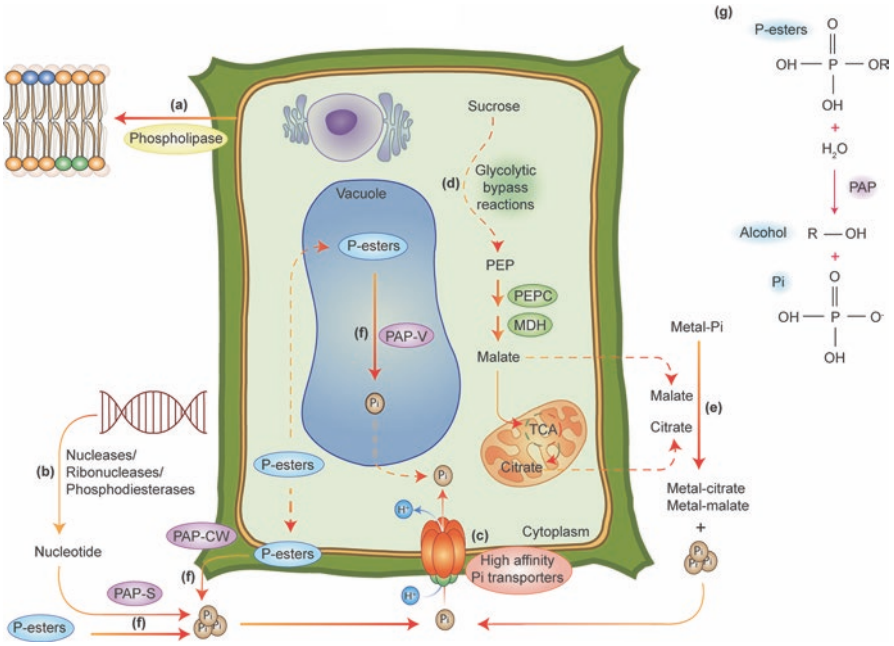

Fig. 2.3 A model outlining adaptive metabolic processes that help plant cells acclimate to nutritional Pi deficiency (Plaxton and Tran 2011). (**a**) Phospholipase induction is accompanied by the replacement of membrane phospholipids (orange sphere) with non-P containing amphipathic sulfonyl and galactolipids (blue and green sphere, respectively). (**b**) Secreted nucleases, ribonucleases, phosphodiesterases, and PAPs participate in systematic Pi mobilization from soil-localized nucleic acids. (**c**) Upregulation of high-affinity Pi transporters of the plasma membrane. (**d**) Alternative pathways of cytosolic glycolysis, mitochondrial electron transport, and tonoplast H^+-pumping facilitate respiration and vacuolar pH maintenance by Pi-deprived plant cells. (**e**) Organic acid anion excretion solubilizes mineralized forms of Pi and organic P as well as increases the ability of secreted PAPs to scavenge Pi from soil-localized organic Pi monoesters. (**f**, **g**) Upregulation of intracellular, cell wall (CW), and apoplast/rhizosphere targeted PAPs enhances the Pi acquisition and use efficiency of Pi-deprived plants (Tran et al. 2010b; Wang et al. 2014). PAPs catalyze Pi hydrolysis from a broad and overlapping range of Pi monoesters with an acidic pH optimum and function in the production, transport, and recycling of Pi

Pi-replete seedlings. AtPAP12 and AtPAP26 function likely includes scavenging Pi from 3′-(d)NMPs derived from nuclease-mediated nucleic acid hydrolysis (Fig. 2.3). This was supported by the impaired development of *atpap12/atpap26* T-DNA double insertion mutant seedlings during growth on media containing salmon sperm DNA as their sole source of exogenous P (Robinson et al. 2012b). Vacuolar and cell wall-targeted AtPAP26 were also strongly upregulated by senescing leaves of Pi-replete plants to remobilize Pi from endogenous Pi-ester pools (Robinson et al. 2012a; Shane et al. 2014). Senescing leaves of an *atpap26* T-DNA mutant exhibited a >90% decrease in APase activity, impaired Pi remobilization, and delayed senescence (Robinson et al. 2012a). The collective results have defined AtPAP26 as a principal contributor to intra- and extracellular APase activity, and that AtPAP26

loss of function elicits dramatic effects on Arabidopsis Pi metabolism that cannot be compensated for by any other AtPAP isozyme. As outlined below, however, several animal and plant PAP isozymes that are secreted into the ECM effectively hydrolyze Pi from phosphoamino acid and phosphoprotein substrates, suggesting that they might function *in planta* as a phosphoprotein phosphatase rather than as nonspecific scavengers of Pi from organic P molecules. However, this discussion first warrants a summary of recent and compelling evidence for extensive and dynamic extracellular protein phosphorylation networks in the animal and plant kingdoms.

2.2 Extracellular Protein Phosphorylation Networks of Animals and Plants: The Neglected PTM

Reversible protein phosphorylation is the most important posttranslational modification (PTM) of eukaryotic proteins since it participates in the control of virtually all aspects of cell physiology and development including signal transduction, cell differentiation, cytoskeleton organization, active transport (ion pumping), gene expression, disease and stress responses, and metabolic fluxes (Moorhead and Tran 2006). Phosphoproteomic studies indicate that phosphorylation occurs in at least 70% of all eukaryotic proteins, with the majority having multiple phosphorylation sites. Protein kinases and phosphatases catalyze the covalent incorporation or hydrolysis, respectively, of Pi groups on target proteins.

2.2.1 Animals

The occurrence of extracellular protein phosphorylation was debated for many years, despite the fact that casein, a secreted storage protein of a mother's milk, was the first phosphoprotein to be discovered over 130 years ago. Numerous secreted proteins[1] have since been shown to be phosphorylated in vertebrate and invertebrate animals (Yalak et al. 2014). For example, phosphoproteomic screens have identified 25 to 85 different phosphoproteins in different human body fluids including cerebrospinal fluid, blood plasma, and saliva. Further studies detected over 500 phosphoproteins in human serum (Yalak et al. 2014), whereas over 1000 animal phosphoproteins listed in the PhosphoSitePlus database (https://www.phosphosite.org) have been annotated as being extracellular or transmembrane proteins (Klement and Medzihradszky 2017). A remarkable feature of the mammalian phosphoproteome (and possibly plant; see below) is that a substantial proportion of the ECM

[1] Proteins occurring in the luminal side of the ER or the Golgi, as well as interior of digestive vacuole (i.e., lysosome in animals, cell vacuole of plants), are also considered to be part of the secreted proteome (i.e., the "secretome").

proteome of cultured or primary tissue samples contain phosphotyrosine (P-Tyr), in addition to phosphoserine (P-Ser) and phosphothreonine (P-Thr) (Bordoli et al. 2014).

In the classical secretion pathway, a short transit peptide targets the protein to the endoplasmic reticulum (ER) and then the Golgi network after which it is packaged into secretory vesicles that are released into the ECM (Fig. 2.4) or targeted to the lysosome. During this transport, the protein may undergo various PTMs apart from glycosylation (a typical PTM of secreted eukaryotic proteins), including dithiol-disulfide interconversion and phosphorylation (Canut et al. 2016; Ghahremani et al. 2016). Proteins of the secretory pathway that are not fully secreted may also be phosphorylated (e.g., the extracellular domain of plasma membrane-spanning receptors). Proteins lacking a transit peptide and thus not entering the classical ER/Golgi secretory pathway can also be exported to the ECM by unconventional means, i.e., via exocytosis or direct translocation across the plasma membrane. Phosphorylation of secreted proteins may precede their export or occur post-secretion via extracellular protein kinases (Fig. 2.4) (Klement and Medzihradszky 2017; Yalak et al. 2014).

Secreted protein kinases, evolutionarily and structurally distinct from cytoplasmic kinases, have been identified in the ECM of mammals, as well as several invertebrate animals (Gerson-Gurwitz et al. 2018; Sreelatha et al. 2015; Tagliabracci et al. 2013; Yalak et al. 2014). For example, Fam20C is a ubiquitous mammalian serine kinase dedicated to phosphorylating a wide range of secreted and highly acidic milk-, salivary-, enamel-, dentin-, and bone-specific proteins (which typically contain an S-x-E-pS consensus motif) involved in diverse processes such as biomineralization (i.e., bone and tooth formation), lipid homeostasis, wound healing, cell adhesion, and cell migration (Sreelatha et al. 2015; Tagliabracci et al. 2013, 2015; Yalak and Vogel 2012). Fam20C resides inside the Golgi but also occurs as an N-terminally truncated, fully secreted form. FAMK-1, a secreted Fam20C ortholog, contributes to fertility, embryogenesis, and development in the nematode worm *Caenorhabditis elegans* (Gerson-Gurwitz et al. 2018; Tagliabracci et al. 2015). In addition, "vertebrate lonesome kinase" (VLK) is a novel secreted protein kinase of mammals that phosphorylates a broad range of ECM proteins on tyrosine residues and is vital for embryonic development (Bordoli et al. 2014; Tagliabracci et al. 2015). High VLK expression occurs in platelets, where it is rapidly and quantitatively secreted in response to specific stimuli. Besides phosphorylating substrate proteins within the Golgi, secreted VLK also phosphorylates tyrosine residues in various protein targets in the ECM using endogenous secreted ATP[2] sources (e.g.,

[2] The protein kinase co-substrate ATP cannot passively diffuse across the plasma membrane owing to its high charge. Thus, extracellular ATP originates from cytosolic ATP via its regulated secretion in the absence of cell lysis; that is, cytoplasmic vesicles laden with ATP secrete their cargo into the ECM of animal and plant cells via exocytosis (Bordoli et al. 2014; Chivasa and Slabas 2012; Yalak and Vogel 2012). It is notable that extracellular ATP is an important stimulus for cell signaling that functions in many aspects of animal and plant physiology, including growth, development, and stress responses (Cao et al. 2014; Chivasa and Slabas 2012; Yalak and Vogel 2012).