

## Plant Protein Kinase and Protein-Protein Interaction

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### Abstract

External and internal stimuli trigger the response of signals in the cell which amplified and communicated by various complex signal transduction networks are mostly initiated with the activation of different receptor proteins. These signaling networks perform as protective strategy to allow cells to cope with external stresses that usually restrain the plant growth and development. Protein kinases are among the most common cellular regulatory components of signal transduction in plants. Protein phosphorylation plays prominent role in various biochemical pathways in plants as a result of active implication in the regulation of cell growth and development. Therefore, protein kinase becomes the most common target used to improve plant growth and development. Gravity force, touch, light, gradients in temperature, humidity, ions, chemicals and oxygen are among the environmental elements influencing the physiological and biological aspects of plants. Hence, many different plant species adopt and response to different environmental stresses where they develop physiological strategies to generate their optimum growth. Beside the involvement in the signal transduction pathways, protein kinases also encourage the protein-protein interaction events within the cells. Therefore, protein phosphorylation plays pivotal roles in activating the protein kinases and transcription factors in various signal transduction pathways. A process whereby a cell communicates and responds to the stimuli from external environment to alter its growth, development, physiology and morphology is known as signal transduction. This signal transduction process involves a serial of biochemical phenomenon that are performed and conducted by many types of enzymes within the cells. Most of the signals are perceived and recognized at the cell surface. The integration and interaction of various signaling information and activities are important, for example, addition of a phosphate to a protein kinase to target the protein substrate. Hence, modification of protein forms protein-protein interaction useful for signal transduction in plant growth and development.

**Keywords:** Protein kinases; Signal transduction; Protein phosphorylation; Protein-protein interaction; Yeast two-hybrid system; Yeast mating

### Protein Phosphorylation and Dephosphorylation

Protein kinases are among the most common cellular regulatory components of signal transduction. Plants are always subjected to various ranges of abiotic and biotic stresses. Plants are sessile natures which are capable of adapting and coping with many challenging environmental conditions with the aids of different protein kinases and hormones to orchestrate growth and development. Light, nutrients, gravity force, water tensions, turgor pressure, organic metabolites, soil quality, mechanical tension, air, growth regulators, pH, carbon dioxide, oxygen, wounding, diseases and electrical fluxes are among the internal and external stimuli that convey information to the plants and plants response by generating signals that are carried by the circulatory system that consists of xylem and phloem. Plant organs usually utilize variety of environmental cues to guide their growth. Young plants are capable to perceive changes in their growth. Stress transport orientation triggers the corresponding physiological and biochemical signals which are then translated into a complex growth response allowing them to straighten up and resume growth. These signals promote cells differentiation that is responsible for growth and development. On the other hand, lower plants and algae exhibit single-cell growth which occurs within the same cell. Thus, different plant species adopt and response to different molecular cytological activities

and they utilize physiological strategies through complex signal networks of protein interactions involving protein kinases to generate their optimum growth [1]. The most studied posttranslational modifications protein is reversible phosphorylation involving two enzymes in two different reactions. Protein kinase in reversible phosphorylation process catalyses the forward reaction whereas protein phosphatase catalyses reverse reaction. Reversible protein phosphorylation is responsible for the control of intracellular activity across all the eukaryotes. Metabolism, contractility, membrane transportation and secretion transcription and translation of genes, cell division and fertilization are orchestrated by the versatile posttranslational modification mechanisms of protein phosphorylation to control various cellular regulatory processes by activating or inhibiting the function of a given protein. Protein phosphorylation and dephosphorylation by protein kinase and protein phosphatase alter the phosphorylated status of protein to switch between the active and inactive states of the protein by direct recognition of the specific sites of protein substrates [2].

Protein phosphorylation and dephosphorylation by protein kinase and protein phosphatase alter the phosphorylated status of protein to switch between the active and inactive states of protein by direct recognition of the specific sites of protein substrates [2]. The structures and functions of many protein kinases and their mechanisms of regulation have been identified. Some are specific for a particular substrate, while others phosphorylate many intracellular proteins [3]. Protein kinase activities are mostly controlled by second messengers,

allosteric effectors and phosphorylation process [3]. Protein phosphorylation and dephosphorylation of serine, threonine and tyrosine residues of a protein triggers conformational changes of proteins results in alteration of their biological properties [3]. The changes of proteins by addition and removal of a phosphoryl group from a protein generate protein-protein interactions, regulate protein stability and modulate enzyme activity [4].

### Protein Phosphorylation Cascade System Function as Regulatory Devices

Reversible phosphorylation involves a reversible shift between modified and unmodified substrate protein which catalyzed by serial of converter enzymes defined as cascade system [5]. Cascade systems are very efficient in the regulation and coordination of reticulated network in metabolic pathways [6]. Through this system, the advantages of signal amplification and flexibility of regulation involving different components in additive, opposite or synergistic systems enable enzymes to sense the fluctuations in the concentrations of substrates and regulatory molecule. These systems are a fundamental part of cellular regulatory devices in animal systems and a mechanism by which neural and hormonal stimuli may be amplified [3,7].

### Some Examples of Plant Protein Phosphorylation

Reversible protein phosphorylation involves a few enzymes in plants. There are a few examples of protein kinases undergo the reversible protein phosphorylation. Pyruvate dehydrogenase multi enzyme complex or known as PDC catalyzes the irreversible oxidative decarboxylation of pyruvate in the glycolysis and the Krebs cycles as well as at the branching point leading to the synthesis of fatty acids and several amino acids. Rao and Randall [8] reported that PDC from different plant tissues was deactivated in an ATP-dependent manner when it was incorporated with  $\gamma^{32}\text{P}$  in a mitochondrial complex. On the other hand, plants commonly accumulate large amounts of alicyclic acids shikimate and quinate [QA]. QA may considered as a reservoir of alicyclic acid that involves the enzyme known as quinate  $\text{NAD}^+$  oxidoreductase [QORase], a type of protein kinase [9]. The QORase particularly from carrot cells only phosphorylates in the presence of calcium and calmodulin. QORase is therefore regulated through a two-cycle cascade system of reversible protein phosphorylation [10]. The protein phosphorylation of QORase was regulated by the changes of calcium content in green leaves where QA contributes 10% of dry weight in green leaves [10].

Another example of plants protein phosphorylation, pyruvate Pi dikinase [PPDK] which catalyzes a double transphosphorylation before giving rise to the formation of phosphoenolpyruvate [PEP], a  $\text{CO}_2$  acceptor in C4 and CAM plants. Burnell and Hatch [11] reviewed that during the protein phosphorylation, the active and unmodified PPDK was phosphorylated on the histidine residue of a protein substrate. Then, the phosphor-intermediate of phosphorylation became a substrate for a converter enzyme to inactivate the PPDK through an ADP-dependent manner [11]. The use of different  $^{32}\text{P}$ -labelled ADPs has shown that it only phosphates the  $\beta$ -position on the adenylate which is transferred onto a threonine residue of enzyme during the inactivation of PPDK. The plants protein phosphorylation occurs in the C4 and CAM plants when PEP carboxylase or known as PEPC catalyses the first step of the carbon dioxide fixation. This enzyme allows the plants to accumulate malate at night [12]. The

PEPC extracted from CAM plants during the light period is inhibited by malate. However, it is usually activated by glucose-6-phosphate. Therefore, the post translational modifications of native PEPC are controlled by light. In other words, the reversible protein phosphorylation is the cause of the changes in the regulatory properties of PEPC.

### Protein Phosphorylations in Different Parts of Plant Organelles: Cell Nuclei

In the isolated nuclei, proteins are easily phosphorylated and the phosphorylation processes are usually catalyzed by endogenous protein kinase [13,14]. Some examples involving the protein phosphorylation in plant nuclei have been identified and correlated with different physiological events in plant system. Chapman et al. [15] and Van Loon et al. [16] proved that there were changes in the chromatin phosphorylation patterns during barley germination and after abscisic acid treatment of *Lemna minor*, thus demonstrating the diverse occurrence of the phosphorylation process during development [15]. On the other hand, Melanson and Trewavas [17] emphasized the relation between cell division and nuclear protein phosphorylation in artichoke tuber. There are examples of direct correlation of protein phosphorylation with gene expression in eukaryotes. The protein phosphorylations in nuclei have been studied in the crown gal tumors of higher plants, which synthesize large amount of RNA during growth phase. For example, the potato tubers that transformed by *Agrobacterium tumefaciens* have the chromatin-bound protein kinases in the chromatin protein phosphorylation which are activated and enhanced concomitantly along with the dramatic increase in RNA synthesis in the growing tumors [18]. Datta et al. [19,20] obtained potentially important finding regarding the regulation of nuclear protein phosphorylation in pea. Highly purified nuclei with their inner and outer membranes were isolated from etiolated pea plumules. When  $\text{Ca}^{2+}$  was added to the nuclear preparation, it increased the level of protein phosphorylations of several nuclear proteins. However, low concentrations of calmodulin antagonists inhibited the calcium-induced response. Red light induced at least three protein phosphorylations while red light inhibited the process in the presence of  $\text{Ca}^{2+}$ . The photo-reversibility of the response by red and far red light indicates that phytochrome is likely to be photoreceptor. Because of the phytochrome is known to regulate the pea gene expression, therefore nuclear protein phosphorylation may be the important step between photo-activation of phytochrome and gene expression. With this result, it provides better understanding of the role of plants protein phosphorylation to external stimuli such as light [19,20]. In conclusion, protein phosphorylation occurs in the plant nuclear is usually activated by metabolism [6].

### Protein Phosphorylations in Different Parts of Plant Organelles: Mitochondria and Chloroplasts

Mitochondria contain protein kinases which possess  $\text{Ca}^{2+}$ /calmodulin and cAMP independent protein kinases that phosphorylate different endogenous polypeptides in an ATP-dependent manner [21]. Some proteins found in plastids can be extensively phosphorylated. Macherel et al. [22] proved that proteins from amyloplasts from sycamore cells could be reversibly phosphorylate in calcium/calmodulin-dependent condition. In tobacco and carrot cells, the regulation of starch metabolism in non-photosynthetic cells through reversible protein phosphorylation had been identified by Bocher et al. [23]. Up to ten protein

phosphorylations were detected in the envelope membrane fraction of chloroplast of pea. From the study, most of the labeled proteins were localized in the outer envelope of nucleus suggested that there was possible role of substrates phosphorylation in the regulation of functional interactions between chloroplast and other compartments [24]. Phosphorylated ribosomal proteins, protein kinase and phosphoprotein phosphatase activities had been detected in stroma of spinach chloroplast. Soll and Buchanan [25] classified a protein kinase found on the outer membrane of spinach chloroplast that able to phosphorylate a stromal protein known as rubisco. This kinase is insensitive to light, cAMP, thioredoxin and plastoquinone however, it is slightly activated by  $\text{Ca}^{2+}$  and independent to exogenous calmodulin [26]. The protein phosphorylation of the rubisco by this kinase was fully activated in the presence of calcium and phospholipids [26]. Several thylakoid proteins are easily phosphorylated *in vivo* or *in vitro*. For example, the phosphorylation of light-harvesting chlorophyll [LHCP] a/b-binding complex is catalyzed by a light-dependent protein kinase [27]. The light effect is mediated by plastoquinone, and thus, when the pool of plastoquinone is reduced, the protein kinase is activated. On the other hand, the phosphorylated LHCP is reversibly hydrolyzed by a light-insensitive phosphoprotein phosphatase which is bound to the thylakoid membrane [28]. When LHCP is phosphorylated, light energy is transferred to photosystem I, whereas the distribution of excitation energy is transferred to photosystem II when LHCP is dephosphorylated. Briantais et al. [29] reported that by controlling the redox state of the plastoquinone, the imbalance of energy is transferred between two photosystems that can be monitored and hence, it improved the efficiency of noncyclic electron transport in the thylakoid membrane.

### Protein Phosphorylations in Different Parts of Plant Organelles: Plasma Membranes

Protein phosphorylation of the membrane-bound proteins may regulate the structure and function of membrane components. Some membrane-associated protein kinases phosphorylate the membrane proteins in plant tissues [30]. Some membrane fractions from pea are able to phosphorylate and dephosphorylate their constitutive proteins. Brummer and Parish [31] demonstrated that majority of phosphorylated membrane proteins are belonged to glycoproteins. Polya et al. [32] demonstrated that the protein phosphorylation which occurred in a well-defined plasma membrane fraction was detected in the endosperm cells of *Lolium multiflorum*. They proved that the plasma membrane-enriched fractions of *Lolium multiflorum* had few polypeptides which subjected to the  $\text{Ca}^{2+}$ -dependent phosphorylation. Besides that, Teulieres et al. [33] showed that protein phosphorylation was highly calcium/calmodulin-dependent in the tonoplast of Acer cells. Through their observation, the changes in the membrane protein phosphorylation level reduced the changes in the electrostatic charges and subsequently reduced the permeability and functions of the transport systems and membrane-bound enzymes activities in plants [33].

### Protein Kinase of Phosphorylation

The phosphorylation and dephosphorylation of proteins serve as a key regulation of cell function and require the protein kinases and phosphoprotein phosphatase to function properly [34]. Protein kinases are often regulated by specific effector compounds or messengers, those as involved, including cAMP-dependent protein

kinases, calcium-dependent protein kinases, cGMAP-dependent protein kinase and a double-stranded RNA-dependent protein kinase. On the other hand, phosphoprotein phosphatases are apparently regulated by a substrate where a given metabolite or metal ion combines with the phosphoenzyme substrate to produce a 'better' or 'poorer' substrate for phosphatase. Protein phosphorylations encourage the protein-protein interactions. Therefore, protein kinases are the primary factors in process of signal transductions. It is possible that phosphorylation at one site may also regulate the dephosphorylation of another different sites. Commonly, the amino acid residues to which the phosphoryl group is transferred in the protein phosphorylation process is usually a serine, a tyrosine or a threonine but occasionally protein kinases catalyze the transfer of phosphoryl group from ATP to histidine or lysine residues in proteins [35]. The protein kinase reactions themselves were reversible as first discovered by Rabinowitz and Lipmann [36]. Lerch et al. [37] also investigated a yeast protein kinase that catalyzed the phosphovitin and casein phosphorylation. The other examples of protein kinases as supported by Rosen and Erlichman [38] demonstrated that the autophosphorylation of type II cyclic AMP-dependent protein kinase was reversible. In order for protein phosphorylation-dephosphorylation reactions to function in the proper regulation, appropriate signals transduction apparently must bring changes in the relative concentrations of phosphorylated and non-phosphorylated forms of protein substrates. For the reversible phosphorylation to occur properly, the control of protein kinase and phosphoprotein phosphatase as well as the simultaneous regulation of the phosphorylation reaction must appropriately perform to alter the ratio of protein kinase to phosphoprotein phosphatase in the right manner within the cells [34]. In other words, protein kinase activity is usually counterbalanced by the action of specific protein phosphatase within the cell.

### Protein Kinases in Various Organisms

Protein kinases are important element in the metabolisms of cell because they are the key regulators of cell function that contribute one of the largest and most functionally diverse gene families across all eukaryotes and prokaryotes by adding phosphate groups to the substrate proteins. Protein kinases direct the function, encourage the protein-protein interactions, regulate protein stability and orchestrate overall activities of cellular processes. Protein kinases play a prominent role in the signal transduction process and involve as coordinator in the complex cell functions particularly in cell cycle. Protein kinases are ubiquitous enzymes and represent one of the largest enzyme families in various organisms. For example, Manning et al. [39] reported that human genome appears to encode 518 protein kinases and of the 518 human kinases, 478 are grouped into a single superfamily which their catalytic domains are related in sequence. And then, these protein kinases are clustered into groups, families and sub-families based on the sequence similarity and biochemical function [39]. Of the 25,706 genes in *Arabidopsis thaliana*, 1,049 proteins belonged to the protein kinases superfamily, sum up to around 4% of the plant's genome [Arabidopsis Genome Initiative, 2000]. According to Manning et al. [39], of all the 6,144 yeast *Saccharomyces cerevisiae* genes, 130 genes encoded the protein kinases. Adams [40] found out that 319 of 18,266 genes in fly *Drosophila melanogaster* are belonged to protein kinases superfamily. Manning et al. [39] detected that of 18,266 genes in worm *Caenorhabditis elegans*, 454 genes encoded the protein kinases. In 2004, Caenepel et al. [41] discovered that the mouse genome appears to encode the 540 protein kinases. 510 of 540 protein kinases have

orthologs in human kinome. According to Goldberg et al. [42], 285 protein kinases are found in *Dictyotellium's* genome and 46 protein kinases are conserved in all of the organisms. Of all the organisms mentioned, approximately 2% of their genomes are corresponding to protein kinases superfamily, except for *Arabidopsis* genome where protein kinases are made up to 4% of the plant's genome. Different percentages of protein kinase genes are present in various organisms. According to Manning et al., [39] and Goldberg et al., [42], 2.12% and 2.28% of the total genes of 6,144 and 12,500 of *Saccharomyces cerevisiae* and *Dictyotellium discoideum* are belonged to kinase genes, respectively. As many as 319 genes from 18,266 of total genes of *Drosophila melanogaster* are belonged to protein kinase according to Adams [40]. On the other hand, Manning et al., [39] stated that 454 genes of 18,266 of total genes are grouped under protein kinase from *Caenorhabditis elegans*. 1,049 protein kinase genes of 25,706 total genes are found in *Arabidopsis thaliana* according to the Arabidopsis Genome Initiative, 2000.

### Conserved Catalytic Domains of Protein Kinase

The diversity of essential functions mediated by protein kinases is shown by the conservation of different 50 distinct kinase families between yeast, invertebrate and mammalian kinomes. The protein kinases are clustered into groups, families and sub-families which the catalytic domains are related in sequence and similarity in biochemical roles. Protein kinases superfamilies have been divided into two distinct subdivisions, the protein serine/threonine kinases and protein tyrosine kinases. The protein serine/threonine kinases phosphorylate the serine/threonine residues of a substrate protein whereas the protein tyrosine kinases phosphorylate only tyrosine residue of a protein. They are grouped according to the amino acid residue of a substrate protein to which the protein kinases transfer their phosphate group to. Similar group of protein kinases has the common characteristic and basic identical mode of action appeared on their catalytic domains [43]. The catalytic domain of the protein kinase of the superfamily constitutes of 250-300 amino acids which carries three different roles in protein phosphorylation. The binding and orientation of the ATP as a complex with divalent cation, for example  $Mg^{2+}$  or  $Mn^{2+}$ , binding and orientation of protein substrate and transfer of phosphate from ATP to hydroxyl groups [Ser, Thr or Tyr] of protein substrates [44]. The kinase catalytic domain consists of 12 conserved sub-domains that fold into a common catalytic core structure. The overall kinase domain folds into 2 lobes structures, the smaller N-terminal and larger C-terminal lobes. The smaller N-terminal lobe constitutes of sub-domains I to IV predominantly involved in the anchoring and orienting ATP. On the other hand, the larger C-terminal lobe which constitutes of sub-domains VIa to XI involved in the substrate binding and phosphotransfer [45]. According to Johnson et al. [45], the sub-domain V of protein kinase spans the two lobes, where the deep cleft between the two lobes is the site of catalysis. The induced fit mechanism which occurs in between the two lobes is responsible for the substrate binding [45]. The N-terminal boundary of protein kinase domain starts seven positions upstream of the first glycine residue of the consensus motif of sub-domain I, GXGXXGXV which the second glycine [G] is invariant. The lysine [K] in the sub-domain II [AXK motif] is essential for the maximal kinase activity which responsible for the anchoring and orienting ATP by the interaction with  $\alpha$  and  $\beta$ -phosphates. The invariant lysine residue forms a salt bridge with nearly invariant glutamate [E] in sub-domain III. The sub-domain IV and V contain no invariant or nearly invariant residue and sub-domain VIa appears to act as support structure. The consensus motif

of HRDLKXXN in sub-domain VIb contains the invariant aspartate and asparagine amino acids. The loop containing the invariant aspartate [D] is assigned as catalytic loop to attract the proton from hydroxyl groups of substrates during phosphotransfer process. The lysine in the catalytic loop helps in the facilitation of phosphotransfer by neutralizing the negative charge of the  $\gamma$ -phosphate during phosphotransfer. The side chain of invariant asparagine [N] in the consensus motif forms hydrogen bonding with the backbone carbonyl group of aspartate as catalytic base to stabilize the catalytic loop and chelate the secondary metal ion. The highly conserved DFG motif in the sub-domain VII lies in the loop that is stabilized by a hydrogen bond between aspartate and glycine. The purpose of aspartate of DFG triplet helps in the  $\gamma$ -phosphate orientation during phosphotransfer.

In the sub-domain VIII, it possesses the APE sequence motif. This sub-domain folds to form a loop facing the catalytic cleft. The nearby invariant glutamate [E] forms an ion pair with an arginine [R] in the sub-domain XI to stabilize the C-terminal lobe. The sub-domain XI possesses a nearly invariant aspartate [D] residue in the consensus motif, DXWXXG to stabilize the catalytic loop. Sub-domain X is poorly conserved whereas the sub-domain XI extends to the C-terminal end of the kinase domain. According to Canagarajah et al. [46], the mitogen-activated protein kinase [MAPK] family member, ERK2, the conformational changes in this MAPK insertion region at the C-terminal of sub-domain X have a regulatory role. The region between the conserved DFG motif in sub-domain VII and APE sequence motif in sub-domain VIII is assigned as the activation segment. According to Canagarajah et al. [46] and Cano and Mahadevan, [47], in the TXY motif of the MAPK family, this is a site where family members are phosphorylated by their protein regulators. In the conformation of inactive kinases, interactions between the DFG region and N-terminal lobe keep the lobes open. The conformation of the segment between the DFG and APE sequence motifs trigger the correct disposition of two kinase domain lobes for activation. The interaction with the substrate also depends to the defined conformation of the activation segment, a crucial role in the substrate recognition [2].

### Classification and Function of Plant Protein Kinases

According to Hanks and Hunter [44], plant protein kinases have been classified into superfamily of five main groups using a phylogenetic analysis based on the alignment of protein kinase catalytic domains. Hanks and Hunter [44] classified the plant protein kinases superfamily into:

- (a) "AGC" group consists of kinase families of cyclic nucleotide-dependent kinases [PKA and PKG], calcium-phospholipid-dependent kinase [PKC],  $\beta$ -adrenergic receptor kinase [ $\beta$ ARK] and ribosomal S6 kinase,
- (b) "CaMK" group consists of calcium/calmodulin-dependent kinase [CDPK] and sucrose-non-fermenting/AMP activated protein kinase [Snf1/AMPK],
- (c) "CMGC" group consists of kinase families of cyclin-dependent kinase [CDK], mitogen-activated protein kinase [MAPK], glycogen synthase kinase [GSK-3] and casein kinase [CK2],
- (d) "Conventional PTK" or known as conventional PTK protein tyrosine kinase group and
- (e) "Other" group that are not falling into the major groups.

This classification is grouping the protein kinases into the families with the similar sequences as well as into biological function-related group. The protein kinase groups are subdivided into subfamilies with similar topology. They frequently display analogous modes of regulation and substrate specification.

#### (a) “AGC” Group

The AGC group is represented by families of cyclin nucleotide dependent kinase [PKA and PKG], calcium-phospholipid-dependent kinase [PKC],  $\beta$ -adrenergic receptor kinase [ $\beta$ ARK], ribosomal S6 family and other close relative members [44]. Member of these kinases undergo protein phosphorylation of Ser/Thr residues of protein substrate in close proximity to the basic amino acids, lysine and arginine. The PKA, PKG and S6 kinases phosphorylate basic residues in specific positions at the N-terminal to the phosphate acceptor site. On the other hand, PKC and PKB also termed as RAC kinase [48] or Akt kinase [49] represents a family of 3-phosphoinositide-regulated serine/threonine kinases [50]. PKB phosphorylate the serine/threonine of protein substrates with basic residues on both sides of the acceptor site. The G-protein coupled receptor kinases [ $\beta$ ARK and relatives] prefer the phosphorylation of synthetic substrate residues within an acidic environment. A common characteristic of this group is that they are regulated by second messengers such as cAMP, cGMP, diacylglycerol and  $Ca^{2+}$  [51]. The first plant protein kinase cloned, PVPK-1 is similar to PKA [52]. The PVPK-1 family is a 70 to 90 amino acid insert between two of the conserved catalytic sub-domain. In plants, PvK1 [bean protein kinase homolog], OsG11A [rice protein kinase homolog], ZmPPK [maize protein kinase homolog], AtPK5, AtPK7, AtPK64 [*Arabidopsis* protein kinase homologs], atp1 and atp2 [*Arabidopsis* protein kinase homolog] and PsPK5 [pea protein kinase homolog] are belonged to AGC group of protein kinase [53].

#### (b) “CaMK” Group

The CaMK group of protein kinase is represented by families of calcium/calmodulin-dependent [CDPK] and sucrose-non-fermenting/AMP activated kinase [Snf1/AMP] [44]. Most of the members of CaMK group are activated by binding of calcium or calmodulin to a small domain at the C-terminal to the catalytic domain. Generally, the members of CaMK group such as CDPK and AMP-activated protein kinase [AMPK] appear to phosphorylate substrates containing basic residues. They prefer a substrate with basic residues at the N-terminal to the acceptor site whereas other members, for examples, elongation factor kinase 2 [EF2K] and phosphorylase kinase [PhK] prefer sites with basic residues located at both N- and C-terminal to the acceptor site of substrate. Regulation by second messengers is common for this group of protein kinase. Calcium-dependent and calmodulin-independent CDPKs are the predominant calcium-dependent protein kinase. Besides that, Snf1 homologs have been identified in plants.

### Family of Calcium/Calmodulin-Dependent Protein Kinases [Cdpks] and other Close Relative Members

CaMK is the protein kinase that regulated by the calcium and calmodulin concentrations. One of the subfamily of CaMK, the protein kinase with intrinsic calmodulin-like domain is known as calcium/calmodulin protein kinase [CDPK]. CDPK in the kingdom Plantae that belongs to Phylum *Angiospermophyta* has a C-terminal calmodulin-like region and an auto-inhibitory region that involved in

the regulation of kinase activity. The C-terminal region of CDPK possesses homology to the calmodulin with four helix-loop-helix calcium-binding sites. This is a junction that joins kinase and calmodulin-like domains which may function as an auto-inhibitory site. The junction covers the active sites during the absence of  $Ca^{2+}$ , however, the site exposes and activates the catalytic domain when  $Ca^{2+}$  concentration increases. During the activation of kinase domain, the binding of the  $Ca^{2+}$  alters the conformation structure of the protein kinase and thus allowing the catalytic activity to commence [44]. Another examples of plant kinases with intrinsic calmodulin-like domain include AtAK1 [*Arabidopsis* CDPK homolog], OsSpk [Rice CDPK homolog] and DcPk431 [Carrot CDPK homolog] [44]. There were several other CaMK involved in  $Ca^{2+}$ -activated signal transduction pathways in plants. CDPKs may also phosphorylate the plasma membrane proton-ATPase and membrane transporters especially those are found attached to the cytoskeleton, plasma membrane and cytoplasm.

### Family of Sucrose-Non-Fermenting/AMP Activated Kinases [Snf1/Ampks] and other Close Relative Members

One of the examples of serine/threonine kinases presents in this family is known as sucrose-non-fermenting /AMP activated kinase [Snf1/AMP]. This kinase has a predominant role in the glucose repression found in *Saccharomyces cerevisiae*. Halford and Hardie [54] and Sheen et al. [55] reported that this enzyme, Snf1/AMPK was responsible as metabolic sensor of the ATP/AMP ratio and involved in the regulation of metabolic flux between anabolism and catabolism by controlling the gene transcription that encoded some enzymes implicated in carbohydrate metabolism. In plants, one wheat kinase of this family known as abscisic acid-responsive protein kinase [PKABA1] which is synthesized when plant cells are induced high concentrations of abscisic acid [44]. There is other plant kinases belonged to this family. PSnf1-RK1N1 is a rye putative protein kinase that complements to yeast Snf1 polarity. PSnf1-AK1N10 is an Arabidopsis putative protein kinase related to SNF1. Another kinase that related to SNF1 is PSnf1-BK1N12 is isolated from barley; WPK4 is the wheat kinase regulated by light and nutrients. Another Snf1 homolog kinase, NPK5 is a functional homologue of yeast SNF1 protein kinase, which plays a central role in catabolite repression in *Saccharomyces cerevisiae*. NPK5 constitutively activates the expression of the glucose-repressible SUC2 gene expression in tobacco. They speculate that NPK5 also plays a role in sugar metabolism in higher plants [44].

#### (c) “CMGC” Group

Cyclin-dependent kinases [CDK], mitogen activated protein kinase [MAPK], glycogen synthase kinase 3 [GSK-3] and casein kinase 2 [CK2] families are belonged to the CMGC group. These CMGC group kinases involved in the downstream of the protein phosphorylation cascade where the region between subdomain VII and VIII is the regulatory protein phosphorylation site known as the “activation loop” [56]. This CMGC group of kinases plays important tasks in the cell metabolism, signal transduction and cell proliferation in eukaryotes. CDKs orchestrate and regulate the different phases of cell cycle in response to the presence of cyclins [44]. For example, tumor progression is always associated with genetic or epigenetic alterations in CDKs or cyclins, which help in sustaining cell proliferation which independence from external mitogenic or anti-mitogenic signals. In

the late 1980s, mitogen activated protein kinases [MAPKs] together with their immediate upstream phosphorylating kinases were discovered and ever since, they became the most studied signal transduction molecules. Generally, MAPKs are essential for role play of many cellular processes which implicated extensively in the regulation of cell proliferation, differentiation and apoptosis across all eukaryotes.

### Family of Cyclin-Dependent Kinases [Cdks] and other Close Relative Members

The members of this cyclin-dependent kinase [CDK] family are activated by cyclins concentrations and have the major responsible for the regulation of cell division. CDKs have a conserved region known as PSTAIRE. Some examples of the CDKs family in plants are Pcdc2, MsCdc2B and OsC2R. Pcdc2 is the homolog of a cell-cycle-controlling gene of flowering plant, Cdc2. MsCdc2B is the Cdc2 cognate gene products that complement G1/S transition. OsC2R is more distantly related Cdc2 homolog isolated from rice [44]. Another family of CMGC is known as mitogen-activated protein kinase [MAPK] family. MAPK has a conserved Thr-X-Tyr [TXY] motif. This MAPK is also known as extracellular-regulated protein kinase [ERK]. ERK is a type of Ser-Thr protein kinase activated by dual phosphorylation on the Thr and Tyr residues of protein substrate. MAPKK is the enzyme of dual specific kinase that phosphorylates Ser, Thr and Tyr residues of protein substrate. MAPKs serve in the regulation of gene expression and cell division. For examples, MAPK homologs from *Arabidopsis*, ATMPK1 and ATMPK2 implicate in the myelin basic protein [MBP] kinase activity. Further evidence showed that the induced of MBP kinase activity in the ATMPK1 and ATMPK2 occurred upon the addition of purified *Xenopus* MAPKK. Plant MAPKs may also serve in cell proliferation where the phosphorylation of MAPK and enhanced activity of MBP kinase increase upon the auxin treatment on plants result the accumulation of putative MAPKK activity [57]. Members of this MAPK family are involved in plant growth and development. MAPKs plays prominent role in the direct regulation of transcriptional factors that activated by a protein kinase cascade consisted of MAPK kinase [MAPKK] and MAPKK kinase [MAPKKK] [58].

### Family of Glycogen Synthase Kinase 3 [GSK-3]

Glycogen synthase kinase 3 [GSK-3] is cytosolic serine/ threonine protein kinase responsible for multiple receptor-mediated intracellular processes. GSK-3 is active in resting condition and behaves as a suppressor of signaling pathways. This metabolic enzyme has remarkable role in the glycogen metabolism. Kaidanovich and Eldar-Finkelman [59,60] reported that GSK-3 acts as a negative regulator of insulin signaling pathway. The abnormal hyperactivity of GSK-3 is presence in the diabetic tissues where it suppresses the function of two important targets of insulin action including glycogen synthase and insulin receptor substrate-1 [IRS-1]. GSK-3 is the factor responsible for the defection of glycogen synthase activity. It also limits the IR-mediated signaling by protein phosphorylation of IRS-1, in other words, GSK-3 phosphorylates IRS-1 and this phosphorylation of IRS-1 impairs the insulin receptor activity in the intact cells [61]. The phosphorylation of IRS-1 can therefore, be perturbed by abnormal over activity of GSK-3 which leads to insulin resistance, may become a potential mechanism in insulin resistance pathway [59,60,62]. And thus, GSK-3 is used as drug discovery target for insulin resistance and Type 2 diabetes. The inhibitors of GSKs bring attention as it can regulate the spread of the diabetes disease [59]. Besides that, GSK-3

may behave as a crucial physiological mediator in the adipose tissue differentiation. The hyperactivity of GSK-3 is detected in the fat tissue of diabetic animals which possesses the adipose accumulation effect during insulin-resistant condition [59]. In vertebrate, the members of GSK-3 family consists of a form of glycogen synthase kinase 3 [GSK 3 $\alpha$ ] and  $\beta$  form of glycogen synthase kinase [GSK 3 $\beta$ ]. On the other hand, the plant GSK-3/ shaggy-related kinases are essential for the flower development, signaling of brassinosteroid, NaCl stress and wound responses [63]. Plants have a multigene family of glycogen synthase kinases [64,65]. One of the GSKs of *Arabidopsis*, AtGSK1 is generated during salt stress and drought condition [66]. When the overproduction of AtGSK1 is provoked by NaCl and abscisic acid, it induces the salt-stress-responsive genes and the accumulation of anthocyanin, and thus, alters the intracellular cation levels which lead to the tolerance of high salt and drought. Another example, a wound-induced GSK-3 in plants known as WIG is activated by wounding in *Medicago sativa* [67]. Upon injury, this WIG protein kinase is rapidly induced the comparatively to transport to the healthy leaves which with low activity of WIG kinase. Other examples of GSKs in plants are *Arabidopsis thaliana* shaggy-related protein kinases or termed as AtSK11 and AtSK12 which appear to be regulated during the development process such as flower patterning at several stages of flower [68]. Both expression of AtSK11 and AtSK12 occur at the periphery of inflorescence meristem and flower meristem. Besides that, ASK $\beta$  is predominantly expressed in the pollen [65]. Other GSKs such as ASK  $\zeta$  and ASK  $\tau$  express during the embryonic development [69]. Despite that, ASK  $\zeta$  is essential during the entire embryo development stages. Plant GSKs are essential regulators for the development of stress and hormone signaling. Brassinosteroids for example, are key regulators of plant growth and development, cell expansion, vascular differentiation, etiolation and reproductive development [70]. In the absence of a brassinosteroid signal, brassinosteroid-insensitive 2 [BIN2] is activated to phosphorylate BRI 1-EMS suppressor 1 [BES1] and brassinazole-resistant 1 [BRZ1] [71-74]. However, during the activation of brassinosteroid signal by brassinosteroid-insensitive 1 receptor kinase [BRI receptor kinase], BIN2 is inhibited which led to the protein dephosphorylation and nuclear accumulation of BES1 and BRZ1 as well as to a subsequent response of brassinosteroid signal [71,72,74]. The identification of nuclear proteins as substrates such as BES1 and BRZ1 for BIN2 indicates that plant GSK are important key regulators in transcriptional regulation. Besides that, GSKs have essential roles in the cytoskeletal proteins and metabolic enzymes regulation in plants [64,65].

### (d) "Conventional PTK" Group

Protein tyrosine kinases [PTKs] are one of the important families of protein kinase in vertebrate. This PTKs family is specific to Tyr residue of protein substrate. In animals, protein tyrosine kinases play pivotal roles in various pathways that regulate cells growth, differentiation and development. For examples, Lck is the lymphoid T-cell protein tyrosine kinase, Blk is the lymphoid B-cell protein-tyrosine kinase, Syk is the spleen tyrosine kinase, EGFR is the epidermal growth factor receptor, ErbB3 is the receptor tyrosine kinase related to EGFR. ErbB4 is the receptor tyrosine kinase related to EGFR. Hek2 is the human embryo kinase, type 2 and Myk1 is the mammary-derived tyrosine kinase, type1 [44]. These PTKs are the protein kinases that specific to tyrosine residue of a protein substrate. The tyrosine-specific protein kinase has the conserved motif of DLR/AAA/RN in sub-domain VIb. In sub-domain VIII, the less conserved motif of PI/VK/RWT/MAPE is

found in the tyrosine kinase specificity [44]. Even though, the conventional PTKs have not been identified in plants, CDK, MAPK and GSK-3 homologs that are regulated by tyrosine phosphorylation in other eukaryotes may also be regulated in plants. Despite that, the absence of the PTKs family does not indicate the lack of tyrosine phosphorylation mechanism occurred in plants. In animals, a majority of PTKs possess the tyrosine kinase phosphorylation. On the other hand, some plants that lack of conventional PTKs, however, some are still performed the tyrosine phosphorylation process may come from dual specificity protein kinase in plants. The conservation regulatory sites of tyrosine kinase present in the plant protein kinases may indicate that the tyrosine phosphorylation is able to contribute as an important physiological role in plants growth and development. For examples, the plant tyrosine phosphorylation is involved in the coordination of the coconut zygotic embryo development [75], *Agrobacterium rhizogenes* infection of roots [76] and phytohormone-stimulated cell proliferation [77]. Osmotic stress in wheat seedlings was shown to be associated with tyrosine phosphorylation [78]. The actin in the contact sensitive plant *Mimosa pudica* is proved to be heavily tyrosine-phosphorylated and the extent of phosphorylation correlates well with the degree of bending of petiole [79].

#### (e) “Other” Protein Kinases Group

In other group, all the protein kinases including many of the cloned plant protein kinases that do not fall into any four families mentioned before are thus grouped together in this “other group”. In this group, the protein kinases contain unique sequences and conserved domain motif that widespread across all eukaryotes.

#### Family of Receptor-Like Kinases [RLks] and other Close Relative Members

Receptors-like kinases [RLKs] play important regulatory roles in the cell differentiation and development by stimulating or inhibiting the cell growth, especially in animal species. However, in plant species such as *Arabidopsis*, the complete genome contains over 600 genes encoding receptor-like kinases [RLKs] responsible in the modulation of gene expression, self-incompatibility response, defense against pathogens and regulation of plant development processes [80]. These RLKs have the N-terminal signal sequence, an extracellular domain that varies in structure, a single membrane-spanning region, and a cytoplasmic protein kinase catalytic domain, a C-terminal cytoplasmic Ser/Thr protein kinase domain (The Arabidopsis Genome Initiative, 2000). All of the plants RLKs phosphorylate serine/threonine residues of protein substrates except to the protein kinases that perform dual specificity activity [81]. In the S-domain class, an S-domain displays a characteristic array of Cys residues and other conserved motifs [81]. Another example of RLKs, leucine-rich-repeat receptor-like serine/threonine kinases or termed as S-RLKs are responsible for self-incompatibility in *Brassica oleracea* [81]. S-RLKs have an extracellular S-domain homologous to the self-incompatibility-locus-glycoproteins [SLG] in *B. oleracea*. The SRLK activity is induced during self-incompatibility. In *B. oleracea*, this S-RLK gene which is linked to the S-locus play an important role as receptor for pollen-derived ligand such as S-locus cysteine rich protein [SCR] during self-incompatibility recognition process between pollen and stigma [82]. Moreover, several S-RLK genes isolated from self-incompatibility may responsible for the plant development. Despite that, one of the S-RLKs of Brassicals is responded in plant defense system [83,84]. Another example of the “other” group subfamily including the *Arabidopsis* ERECTA receptor-

like kinase that involves in organ shape development, CLAVATA1 receptor-like kinase, a regulator of meristem and flower development in *Arabidopsis* regulates cell differentiation at the shoot meristem, HAESA kinase which functions in developmentally regulated floral organ abscission process and BRI1 kinase which involves in brassinosteroid perception [85-89]. Besides that, according to Song and coworker [90], they demonstrated that the rice gene Xa21 provided the rice-specific resistance to *Xanthomonas oryzae*. From all the examples mentioned above, LRR-RLKs play an important role in disease resistance. In addition, one of the LRR-RLKs found in tomato Cf disease resistance gene products, which performed rice-specific resistance to *Cladosporium fulvum* contain extracellular LRR domains with lack of cytoplasmic protein kinase domain [91]. In the tumor-necrosis factor receptor TNFR class, the maize crinkly 4 [CR4] gene product displays TNFR-like repeats that have a conserved arrangement of six cysteines and seven repeats of 39 amino acids that display a weak similarity to the RCC GTPase [92,93]. CR4 kinase is required for the normal differentiation of leaf epidermis [92]. In epidermal growth factor, EGF class, four wall-associated receptor kinase [WAKs], WAK1 to WAK4 have the extracellular domains containing repeated sequence related to mammalian epidermal growth factors is required for pathogenic response [94,95]. In pathogenesis-related [PR] class, the *Arabidopsis* PR5K-like receptor kinase is induced upon infection by microbial pathogens [96]. In lectin class, *Arabidopsis* LecRK1 is the known example of this class, displays an extracellular domain homologous to carbohydrate-binding proteins of legume family [97]. LecRK1 is suggested to be involved in a perception of oligosaccharide-mediated signal transduction process. McCarthy and Chory [93] discovered that *Arabidopsis* genome displays more than 30 genes of lectin-RLKs.

#### Family of Raf-like kinases

Raf-like kinase is the kinase that implicated signal from RTKs seven-transmembrane-domain receptors and cytokine receptors. An example, constitutive triple response 1 [CTR1] may involve in the ethylene triple response that have been proposed as a negative regulator for ethylene signal transduction process [98]. Kieber et al. [98] conducted mutation on CTR1 gene which led to the increased of CTR1 protein kinase catalytic activity during the activation of ethylene response. The N-terminal region of CTR1 contains a negative regulator function which has the significantly weaker homology, even though the catalytic domain resembles the sequences which identified to be Raf family. Tosl [TsI] is also one of the protein kinases of other group. TsI is implicated for the proper initiation and organ primordia development. It may require for the normal floral organs and leaf morphological development in plants especially *Arabidopsis* [99].

#### Dual Specificity Protein Kinases

Most of the signaling pathways regulate all the essential stages of cell growth such as cell proliferation, differentiation and apoptosis by the protein serine/threonine kinases [PSKs] and protein tyrosine kinase [PYKs] of protein phosphorylation. PSKs and PYKs found in the cell are different, PYKs are commonly found in the form of growth factor receptors situated at the plasma membrane or associated closely to transmembrane glycoprotein [100]. On the other hand, the inner surface of plasma membrane cytoplasm and nucleus are the commonly found location of PSKs in the cell. Protein kinases add a phosphate group to a specific amino acid residue. The major

phosphorylation sites of kinases are found in the alcoholic group of serine and threonine as well as at the phenolic group of tyrosine. Serine/threonine [Ser/Thr] kinases phosphorylate serine or threonine residue of a protein whereas the tyrosine kinases phosphorylate tyrosine residue of a protein substrate. To date, a novel discovery of third group of protein kinases that capable of phosphorylating all three hydroxyl amino acids is revealed, an evolutionally breakthrough of a new protein kinase known as dual specific kinases [DSKs]. There are more than 500 protein kinases identified in plants and most of them are belonged to serine/threonine kinase, only one fifth of them are however, belonged to tyrosine kinase, minority belonged to DSKs. DSKs play a critical role in the crosstalk which occurs between PSKs and PYKs. The DSKs share the consensus kinases motif of both Ser/Thr and Tyr-kinases [101].

Commonly, DSKs implicate in a central role in the 'kinase cascade' that regulate cell proliferation, differentiation and apoptosis. Lindberg et al. [101] divided the DSKs into three groups: first, kinases with dual specificity characteristic that phosphorylate Ser/Thr and Tyr residues of exogenous substrates; second, kinases that exhibit dual specificity only by autophosphorylation and lastly, kinases that possess the structural motif characteristic of dual specificity kinases. For example, all the protein kinases have 12 conserved sub-domains in their kinase catalytic domain. When a protein kinase has the KXXN sequence motif in sub-domain VIb, which is indicative of serine/threonine kinase specificity and CW[X]<sub>6</sub>RPXF in sub-domain VIII, which is indicative of tyrosine kinase specificity, suggesting that the identified kinase is belonged to dual-specificity kinase family that phosphorylate serine, threonine and tyrosine residues. DSKs have been implicated in the signaling pathways in eukaryotic cells, new family of signal transducer. The signaling module pathway involving DSKs always comprises of a minimum three kinases which includes an upstream Ser/Thr kinase, middle dual specificity kinases and a downstream of Ser/Thr kinase. For example, in a DSK module pathway, the kinase core consists of a minimum three kinases, an upstream Ser/Thr kinase, middle dual specificity kinase and lastly a downstream Ser/Thr kinase. When external stimulation activates a receptor, the stimulus provokes an upstream Ser/Thr kinase via Ras or Rho family of GTPase. Next, the upstream Ser/Thr kinase provokes the middle DSKs through protein phosphorylation and eventually the phosphorylated DSK stimulates the downstream Ser/Thr kinase which possesses the TXY motif by dual phosphorylation at the Thr and Tyr-residues [47,102-104].

### Dual Specificity Protein Kinases in Plants

Handful of novel DSKs in plants have been reported including *Arabidopsis* protein kinase 1 or termed as APK1 [105], *Arabidopsis* adenosine kinase 1 [ADK1] and adenosine kinase 2 [ADK2] [106,107], protein kinase isolated from *Arabidopsis thaliana* cDNA termed as ATN1 [108], protein kinase isolated from soybean [*Glycine max* L.] named as GmPK6, [109], *Oryza sativa* dual-specificity protein kinases or designated as OsDPKs [110] and protein kinase isolated from root of *Cucumis sativus* [111]. All DSKs have the distinctive roles in the plant metabolism. A cDNA clone from *Arabidopsis thaliana*, coding for a novel DSK known as *Arabidopsis* protein kinase 1 [APK1] is predicted to consist of 410 amino acid residues which the kinase catalytic domain was flanked by short non-kinase domain [105]. The sequence motifs in sub-domains VI and VIII are close related to serine/threonine kinase whereas the motif in sub-domain IX is related to tyrosine kinase thereby APK is the first plant protein kinase that

phosphorylates tyrosine, serine and threonine residues of protein substrate [105]. APK favors the serine and threonine phosphorylations of various protein *in vitro*, the tyrosine phosphorylation however, occurs on only limited types of protein substrates [105]. Hirayama and Oka, [105] suggested that APK1 possesses the characteristic of receptor-like kinase [RLK] whereby the amino acid sequence of its N-terminal portion [G-I-C-L-S-A] is indicative to a sequence encoded for protein N-myristoylation in *Dictyostelium* [G-I-C-A-S-S], and thus, APK1 may associate with membranes and contribute the pivotal role in signal transduction similar to other kinases such as *Zea mays* protein kinase 1 [ZmPK1], *Brassica oleracea* kinase encoded at the self-incompatibility locus and human met kinase [MET].

Another DSK from *Arabidopsis thaliana* known as adenosine kinase [ADK] possesses two types of isoforms, ADK1 and ADK2, both with similar sequences of amino acid and nucleotide, 92% and 89%, respectively [106,107]. These isoforms of ADK1 and ADK2 are involved in the conversion of adenosine [Ado] to adenosine monophosphate. Moffatt et al., [106] reported that both ADK genes expressed constitutively with higher mRNA levels found in the stem and root, whereby ADK1 transcript levels were commonly higher than ADK2. There was apparently substantial ADK accumulation with the highest levels observed in leaves, mature flowers and stems compared to poorly protein expression in roots and dry seeds of *Arabidopsis thaliana*, with very low ADK enzyme activity, suggesting that these areas possessed an inhibitor of ADK enzyme activity [106]. These ADKs protein expression levels in *Arabidopsis* suggested that they are involved in the metabolic role in the salvage synthesis pathways of adenylate nucleotides from Ado, being part of adenylate metabolic network, as well as implicated in the regulation of plant hormone, cytokinin. Ado is the predominant source of transmethylation cycle. Without the ADKs, it could lead to the increase of Ado and reduction of S-adenosyl-Met-dependent transmethylation [112]. ADK is suggested in contributing an important role in sustaining transmethylation reactions where it behaves as a coarse metabolic regulator to reduce the cellular concentration of free adenosine.

Another study by Young and coworker, [107] showed that ADK is a modulator of root cap morphogenesis and gravitropism, whereby increased levels and activity of the soluble ADK are detected in the root tip of *Arabidopsis thaliana* upon gravi-stimulation. ADK allows the plant roots exposal to environmental stresses to deploy growth behaviors that are less dependent to gravitropism, and thereby enable plants to grow in more favorable environments [113]. Increased activities of *in vitro* S-adenosylhomo-Cys hydrolase [SAHH] and ADK activities from leaves extract of spinach was also detected when it was subjected to successively high levels of salt stress [114]. In mammalian cells, ADK is a pivotal kinase in regulating the intra- and extracellular levels of Ado, modulating methyltransferase activity, implicating in the production of polyamines and secondary compounds and being involve in cell signaling process [107]. A novel DSK isolated from *Arabidopsis thaliana* cDNA termed as ATN1 composed of 356 amino acid residues, where its catalytic domain sequence displays a mosaic structure with the motifs based on serine/threonine and tyrosine kinases, found in the sub-domains VIb, VIII and IX. In the sub-domain VIb, ATN1 possesses the sequence motif of DLKPEN corresponds to the consensus sequence DLKXXN found in serine/threonine kinases family. Additionally, the RWMape sequence motif in sub-domain VIII and CW[X]<sub>6</sub>RPXF in sub-domain XI of ATN1 are conferred to consensus sequences K/RWT/MAPE and CW[X]<sub>6</sub>RPXF of tyrosine kinase, respectively. All these sequence motifs indicate that ATN1 is a dual specific kinase, which closely related to a DSK isolated



from soybean, GmPK6. ATN1 does not contain the transmembrane domain, is thereby clearly not belong to receptor-like kinase family [RLKs], however due to the 45-50% identical amino acid residues with a protein kinase isolated from soybean, GmPK6 in their catalytic domain, they are grouped together under the same subfamily of RLK-related group which diverges significantly from RLKs themselves [108]. The amino acid sequence of its N-terminal of ATN1 displays a sequence motif of MGSASGF is indicative to a consensus sequence, MGXXX[S/T/A/G/C/N]X, encoded for N-myristoylation which involves proteins associated with membranes as reported by Towler et al.. This co-translational modification is catalysed by myristoyl-CoA:protein N-myristoyl transferase [NMT], which commonly found in wheat [115]. ATN1 just like other kinases of similar N-myristoylation motif such as tomato protein kinase Pto and Fen, APK1 and PRKINA, are all contain two-short non-catalytic domains and mixed serine/threonine and tyrosine kinase affinities [108]. Since the N-terminal portion of ATN1 bears the same structural relationship to the N-myristoylation which is similar to APK1, both may associate with membranes *in vivo* and thereby contribute to the signal transduction in plants. The presence of ATN1 gene transcripts in plant organs suggested that this DSK is required for normal cellular processes and development as well [108].

The cDNA coding for a novel DSK isolated from soybean [Glycine max L.] termed as GmPK6 which belongs to serine/threonine protein kinase family, also has tyrosine kinase specificity. From their observation, 2.5 kb of RNA transcripts of GmPK6 from various tissues of soybean seedling were detected. Lesser transcript levels were observed in dark-grown soybean tissues such as in roots, epicotyls and hypocotyls except for bud tissues where it was equally abundant to that found in the light-grown soybean [109]. These indicated that the transcription of GmPK6 is tissue-specific and light regulated. GmPK6 is composed of 462 amino acids with a basic 127 amino acid N-terminal which identical to the U1 small nuclear ribonucleoprotein, U1 snRNP 70K protein or U1 70K of *Xenopus laevis*. U1 snRNP 70K protein is intricately involved in both constitutive and alternative splicing of pre-mRNAs [116,117]. Since there is structural similarity at the N-terminal of GmPK6 with U1 snRNP 70K protein, Feng et al., [109] suggested that GmPK6 shares a common evolutionary origin with the snRNP protein which involves in the process of pre-mRNA splicing, and thereby may possibly be a nuclear protein. On the other hand, the 355 amino acid C-terminal of GmPK6 exhibits the mixed of both serine/threonine and tyrosine kinase affinities. The catalytic domains of GmPK6 in the sub-domain VI with HRDLKPEN motif is corresponding to the consensus motifs of classical protein serine/threonine kinase family [44]. The sub-domain VIII with GTYRWMAPE motif and CWSLQPKRPEF motif in sub-domain XI of GmPK6 are indicative of tyrosine kinase specificity, PI/VK/RWT/MAPE and CW[X]<sub>6</sub>RPXF consensus sequences found in the sub-domain VIII and XI, respectively. However, the entire kinase domain of GmPK6 has higher homology with tyrosine kinases compared to serine/threonine kinases due to the present of a single tryptophan at the position 395. GmPK6 together with a protein tyrosine kinase from *Dictyostelium discoideum* or DPYK2 exhibit comparable structural characteristic in that each contains the mixed serine/threonine and tyrosine kinase domains indicating the functional significance in their structural similarities.

Four *Oryza sativa* dual-specificity protein kinases or designated as OsDPK1, OsDPK2, OsDPK3 and OsDPK4 shared the similar homology with known typical plant protein kinases such as GmPK6 from soybean, ATN1 from *Arabidopsis* and NtDSK1 from tobacco

[110]. OsDPK1-4 are not only shares a same homology with these protein kinase but also shows highly absolute conservation of 11 sub-domains in the catalytic domains known to implicate in kinase activity, suggesting that OsDPK1-4 are indeed a protein kinase. In addition, OsDPK1-4 also represented as a dual specific kinase found in the rice genes family. The sequence motif DLKSDN in the sub-domain VIb of OsDPK1-4 is believed to confer to consensus sequences DLKXXN of protein serine/threonine kinases. On the other hand, the sequence motifs RWMape in sub-domain VIII and CW[X]<sub>6</sub>RPXF in sub-domain XI of OsDPK1-4 resemble two strong consensus sequences K/RWT/MAPE and CW[X]<sub>6</sub>RPXF of tyrosine kinase family. These catalytic domains found in different sub-domains of OsDPK1-4 indicate that OsDPK1-4 are belonged to the dual specificity kinase [110], closely related to several other plants DSK such as GmPK6 from soybean and ATN1 of *Arabidopsis*. The expression of OsDPK1-4 varies in various rice tissues where OsDPK1-4 function differently.

Better expression of OsDPKs have been observed in leaves, roots, stems and immature spikes of rice by drought, salinity, ABA and infection of *Maganaporthe grisea* except for OsDPK4 [110]. The OsDPK1-3 protein expression implicates in different aspect of plants signal transduction in dueling with abiotic and biotic stresses. The OsDPK1-3 expression was rapidly induced within 3 to 6 h in rice seedlings treated with exogenous ABA. Further evidences showed many environmental response cis-acting element including ABRE, DRE/C repeat, MYB binding sites and W-box are found in the regions of OsDPK1-3 could suggest their implication in abiotic and biotic stresses. On the other hand, low level of transcript of OsDPK4 was detected in four kinds of rice tissues indicated that lesser expression of OsDPK4 during plant responded to abiotic and biotic stresses. However, it is still remained unclear about the potential role of OsDPK4 in plant signal transduction pathways. Protein kinase isolated from root of *Cucumis sativus* [cucumber] is one of the DSK with 352 amino acid residues with the mixed catalytic motifs of both serine/threonine and tyrosine kinases [111]. Based on the amino acid sequence of the catalytic motif found in this protein kinase, DLKPEN in the sub-domain VIb is conferred to consensus sequences DLKXXN of protein serine/threonine kinases. The sequence motifs RWMape in sub-domain VIII as well as CW[X]<sub>6</sub>RPXF in sub-domain XI of this cucumber kinase are indicative of consensus sequences K/RWT/MAPE and CW[X]<sub>6</sub>RPXF found in the tyrosine kinase family. These bring to the conclusion that this protein kinase shared the serine/threonine and tyrosine kinase activities, a novel DKS isolated from cucumber. The presence of sequence motif of MGSGNGF at the N-terminal of the cucumber kinase corresponds to a N-myristoylation motif with several function such as modification site for glycine at position 2, indication of the presence of either serine, cysteine, or glycine at position 6, absence of praline or charged amino acid at position 3 and absence of praline at position 7 that influence the efficiency of myristoylation process. With this motif, cucumber kinase was suggested to a protein kinase that associated with membranes, similar to ATN1.

A sequence motif of PXXX in the region of C-terminal of cucumber kinase may indicate it is a site for the plant signal transduction to take place with other kinases. The cucumber kinase was highly expressed in root tissue [111]. The expression of the cucumber kinase in root tissue increased threefold at 12 h after the cold treatment and gradually increased to fivefold at 24 h under the same treatment. However, when drought treatment was applied to root tissue after 2 h, the RNA transcript level decreased to half of control and further reduced to

10-20% of control at 10 h of drought treatment. No significant changes of RNA transcript levels were observed when the cucumber roots were exposed to the high salt condition [111]. Taken together, this cucumber kinase is involved in signal transduction pathways in response differently to various abiotic stresses. The cucumber kinase was induced by cold, down-regulated by drought and no response to high salinity. Thereby, cucumber kinase plays pivotal role in the abiotic stresses response pathways. More plant DSKs have been discovered recently. These protein kinases play tremendous roles in all aspects of cell physiology, regulation of transcription factors, cell division, embryogenesis, stem cell development as well as protein translation. The investigation on DSKs has been increasing popular. Many DSKs will be uncovered in years to come. By knowing their general functions in cell metabolism, it allows one to better understand the effects of DSKs in cell growth and development.

### Protein-protein Interaction via Yeast Two-hybrid System

The interactions between proteins are important for numerous biological functions, for example, signals from the exterior of a cell are mediated to the inside of that cell by protein-protein interactions of the signaling molecules via signal transduction process that plays a fundamental role in many biological processes especially in plant system. Most cellular processes in plants are regulated by multi-proteins interaction including regulation of cell division and replication, flowering, fruit ripening and seed germination, photosynthetic regulation, apicals development. Protein-protein interactions are of central importance for virtually every process in a living cell. Therefore, protein-protein interaction study leads to the identification of substrates for protein kinases that in return uncover and disclose the key role of kinases in cells [118]. In conclusion, the information about these interactions improves our understanding of cell regulation, growth and development and thereafter creates healthier plants by manipulating the substrates and protein kinase in various signal transduction pathways.

The protein-protein interaction via yeast two-hybrid system takes advantage of the modular characteristic of GAL4 and eukaryotic transcription factors [119-125]. Many eukaryotic transacting transcriptional regulators are composed of physically separable and functionally independent domains which have at least two distinct functional domains: a DNA binding domain [DBD-BD] binds to operator sequence and an activation domain [AD] activates transcription of certain genes especially reporter genes in yeast two-hybrid system. The DNA-binding domain does not activate the transcription unless it interacts with activating domain. DBD binds the transcription factor to promoter upstream of a target gene and AD then recruits the RNA polymerase II complex to start transcription which will express the downstream reporter gene as indicative of successful interaction of DBD and AD [126-128]. Both domains are required to activate a gene, where two domains are part of the same gene as in yeast GAL4 protein. Physical separation of DBD and AD by recombinant DNA technology leads to a non-functional transcription factor and thus terminates the expression of the downstream reporter gene [128,129].

However, if the DNA-BD and AD are brought close together into close physical proximity in the promoter region, the transcriptional activation function will be restored. In yeast two-hybrid system, 'bait' is constructed by fusing a protein of interest to the DBD and 'prey' is constructed by fusing a second protein to the AD. Bait-expressing

yeast reporter strain receives the nuclear localization signal present in the DBD and thus imports bait to the nuclear. Consequently, the bait binds to the operator sequence upstream of a reporter gene. However, the transcription of reporter gene is not activated as a result of the lacking AD sequences in bait to recruit the RNA polymerase complex to activate the transcription of reporter gene in yeast. On the other hand, even though the prey interacts with the RNA polymerase II to form the RNA polymerase complex, but the expression of the prey does not activate the transcription. The AD-prey fusion is not positioned at the promoter sites upstream of reporter gene due to lack of the DBD positioning the complex at the promoter. Neither the bait nor the prey can activate transcription of the downstream reporter gene when DBD and AD express independently. Thereby, when bait and prey are co-expressed in the same yeast cell, bait and prey interacts with each other, stimulate a functional transcription factor by recruiting the prey to the reporter gene via its interaction with the bait located at the upstream promoter region. The reconstituted "hybrid" transcription factor then activates the downstream reporter gene. In a yeast two-hybrid assay, activation of the reporter gene is measured, for example as growth on a selective medium. The yeast two-hybrid screen is a genetic assay to detect protein-protein interactions *in vivo*. The advantages of using the yeast two-hybrid system include discovery of novel and unknown binding partners of specific proteins by screening cDNA libraries in shorter fraction of time. The system delivers genetic information of cDNA which can be used immediately for further studies. By using the LexA-based two-hybrid system, DBD-target fusion protein is expressed at high levels from the strong ADH1 yeast promoter in pLexA, AD fusion proteins are also expressed at high levels upon induction of the same promoter. The construction of the promoter responsible to drive expression of the reporter genes is important not only for the regulation, but also for the sensitivity and background of this transcription-based assay. In the LexA system, the reporters are under control of multiple LexA operators. This allows several DNA-BD/bait hybrid proteins to bind to each promoter and effectively amplifies the intensity of even weak signals [130]. Thereby, the LexA-based interaction is able to detect specific protein-protein interactions in yeast easily [130,131].

Two different cloning vectors are used to generate fusions of DBD and AD domains to genes which encoding proteins that potentially interact with each other. The DBD is the entire LexA protein including LexA DNA-binding domain. In the LexA-based system, the DBD-target fusion protein is expressed at high levels from the strong ADH1 yeast promoter in pLexA. AD fusion proteins are also expressed at high levels upon induction of the same promoter. The DBD-target fusion protein binds to the LexA operators but fail to activate transcription without the activation domain. In the absence of bait fusion protein, the AD-library fusion protein cannot bind to the LexA operators and thus does not activate transcription. Interaction between the bait and library proteins hybrid *in vivo* creates a novel transcriptional activator with binding affinity for LexA operators [131]. This transcription factor then activates the reporter genes having upstream LexA operators and this makes the protein-protein interactions phenotypically detectable on selective plates. If the two hybrid proteins do not interact with each other, the reporter genes will not be transcribed. The LexA-based two-hybrid system selects the conditional expression of nutritional reporter genes to detect and identify the large numbers of yeast which protein-protein interactions occur. When the positive clones are identified on the selective plate, the immediate action is to screen the clones to identify the genes encoding the interacting proteins of interest.

LEU2 and lacZ are among the reporter genes operate under the control of multiple LexA operators are commonly used as reporter genes in LexA-based two-hybrid system [132]. The upstream promoters differ in the sequences flanking the LexA operators. This sequence dissimilarity helps to eliminate some false positives and to confirm the positive two-hybrid interaction [130,133]. For example, the integrated LEU2 nutritional reporter gene allows the auxotrophic yeast host cell to grow on SD medium lacking leucine when transformed with plasmids encoding interacting hybrid proteins. Most two-hybrid yeast methods also utilize *E. coli lacZ* gene as a second reporter gene. Usually the colonies growing on the selection plates are assayed for the activation of lacZ in a second screening. Screening for expression of lacZ which has a different promoter from LEU2 reporter eliminates many of the false positives that arise in a typical two-hybrid library screening. Yeast strain producing  $\beta$ -galactosidase utilizes X-gal in the medium is the indicative of DBD-target fusion protein [bait] and GAL4 AD-library fusion protein [prey] hybrid interactions which is directly assayed on the nutritional selective plates. The X-gal assay monitors the activation of lacZ reporter gene encoding the secreted enzyme  $\beta$ -galactosidase which hydrolyzes colorless X-gal into a blue end product, forming the blue color colonies on the selective plates supplemented with X-gal. The location of lacZ on an autonomously replicating, high-copy-number plasmid means there are many copies of this reporter gene in each cell. Thus, in the LexA system, it is possible to assay  $\beta$ -galactosidase activity directly on the culture plate by including X-gal in the medium [132].

### Yeast Two-Hybrid System via Yeast Mating

Yeast mating is a convenient way to introduce two plasmids into the same host cells [133,134]. The yeast mating-based screening guarantees the highest coverage of the cDNA library which was up to the coverage of more than  $5 \times 10^6$  independent clones, and thereby the greatest chances of finding weak interactors. The hybrid clones are plated on the selective agar which has been determined in the pilot screening.

Abundant of two-hybrid experiment are utilizing yeast mating method to bring together bait and prey in a diploid strain during library screening. Haploid yeasts fuse to form a diploid when cells of opposite mating type, termed as a and alpha are mixed together. The a-type yeast cells secrete mating pheromone known as a-factor while alpha-type cells secrete alpha-factor. To allow the mating to be successful, MATa strain is mated with MATalpha. The mating type of *S. cerevisiae* is determined by the genetic composition of the MAT locus [135]. MATa haploids express the genes a1 and a2 from the MAT locus while MATalpha haploids express alpha1 and alpha2 from MAT locus [136]. MATa reporter strain of NMY32 was mated with the MATalpha strain of Y187 to produce a diploid which heterozygous at the MAT locus to be able to undergo meiosis and sporulate. However, in the library screening study, the diploids grown on the selective plate should not incubated longer than 2 weeks to avoid the formation of spores when nitrogen became the limiting growth factor [137,138]. The yeast mating is significantly reduced the labor and time involved in performing a two-hybrid library screening. It eliminates the need for library-scale yeast transformations. The optimized mating protocol improves the chances of finding rare and novel protein-protein interactions which lead to more reproducible results. Additionally, the yeast mating method is especially useful to combine reporter genes from different strains. Two haploid strains of opposite mating type with the plasmid markers as markers allow the selection

of the mated diploids on selective plate. In addition, the markers in two yeast reporter haploids were compatible where the reporter gene in one yeast haploid were not complemented by the other haploid strain. Yeast mating significantly reduces the time and the number of transformations required to demonstrate the specificity of the interaction when many clones are being analyzed. When both of the fusion proteins are interacted, the transcription of the reporter gene will be activated to enable the auxotrophic yeast two-hybrid strains to grow on nutritionally selective media. The integration of nutritional reporter genes in the diploids provides a sensitive growth selection which allows identification of positive transformants out of several million candidate clones.

In respect of yeast mating, after 20 h of mating, the formation of zygotes was present. A zygote typically has a three-lobed shape, the lobes representing the two haploid of parental cells and a budding diploid cell [138]. The mating was continued for another 4 h. The mating culture was centrifuged and the cells pellet was resuspended in medium supplemented with antibiotic. Dilution of the mating mixture was plated on control and selective plates. The total number of clones screened was determined using the cfu/ml of diploids calculated after the overnight incubation of the mating culture. The agar plates were incubated at 30°C until colonies appeared generally 3 to 8 days or more to allow slower growing colonies of weak positive interactors. Small and pale colonies which appeared after 2 days of incubation but never grow to >1 mm in diameter should be ignored because true diploids are robust and can grow to >2 mm. Not all of the diploids surviving in this selection agar will be true two-hybrid positives. However, the most common class of false positives can be eliminated by sorting the diploids through passaging on the same selective medium. Other elimination of false positives can be carried out by screening for the expression of all three reporter genes, for examples, ADE2, HIS3, and LacZ [139]. The expression of HIS3 and ADE2 reporter genes can be determined by growing the diploids on the selective agar-his-ade until the colonies at least 1mm in diameter. Additionally, LacZ expression can be performed by subjected the yeast colonies which grown on the selective agar-his-ade to the assay of  $\beta$ -galactosidase activity. Positive colonies obtained in the library screening should not be stored on the same selective plates for more than 2 to 4 weeks to avoid the potential of diploid strains to undergo meiosis and form spores when nitrogen becomes limiting [137,138]. For long term storage, diploids were prepared as glycerol stock and freeze at -70°C.

### Verification of Positive Diploids Using Continuous Passaging and LacZ Expression

To identify the interacting partners, all positive clones are picked, transferred to the same SD medium. The prey clones are passaged for one week to remove any non-specific interactors. After passaging continuously for five rounds, the prey clones are reconfirmed and analysed for LacZ reporter gene using HTX  $\beta$ -galactosidase assay in 96-well plate. The expression stringency of LacZ reporter gene from different preys was categorized based on the  $\beta$ -galactosidase expression. Combination of the continuous passaging with LacZ assay guaranteed that only those interactors which consistently activated expression of HIS3, ADE2 and LacZ reporter genes are proven to be genuine prey interactors. Thereafter, the positive interactors are subjected to sequencing to identify the preys of the bait, to disclose the phenomenon of the protein-protein interaction.

## Conclusion

Protein phosphorylation plays a prominent role in various biochemical pathways in plants and it is involved in the regulation of cell growth and development. Therefore, protein kinase becomes the most common target used to improve plant growth and development. Many protein kinases are isolated in various plant systems, but in vivo physiological roles of kinases in whole plant context are remained imprecise. To date, in depth knowledge about the role of protein kinase is a prerequisite for the development of novel transgenic plants with growth ability against various hostile environments.

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