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REVIEW ARTICLE

# Recent Advances in Substrate Identification of Protein Kinases in Plants and Their Role in Stress Management

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**Abstract:** Protein phosphorylation-dephosphorylation is a well-known regulatory mechanism in biological systems and has become one of the significant means of protein function regulation, modulating most of the biological processes. Protein kinases play vital role in numerous cellular processes. Kinases transduce external signal into responses such as growth, immunity and stress tolerance through phosphorylation of their target proteins. In order to understand these cellular processes at the molecular level, one needs to be aware of the different substrates targeted by protein kinases. Advancement in tools and techniques has bestowed practice of multiple approaches that enable target identification of kinases. However, so far none of the methodologies has been proved to be as good as a panacea for the substrate identification. In this review, the recent advances that have been made in the identifications of putative substrates and the implications of these kinases and their substrates in stress management are discussed.

**Keywords:** Kinase, Post-translational modification, Phosphorylation, Signal transduction, Substrate identification, Stress management.

## 1. INTRODUCTION

Owing to the limitations of sessile lifestyle, plants constantly encounter plethora of adverse environmental stimuli like drought, salinity, harsh temperatures and other biotic and abiotic stresses, which adversely affect their normal growth and development. To ward off such stressful situations, plants have developed an array of sophisticated and elaborate innate mechanisms. After encountering growth-impeding stimuli, plants reprogram their gene expression and physiology and hence display different adaptive responses. A large number of proteins are involved in transducing these external signals into adaptive responses. These important categories of molecules-comprising a diverse array of kinases and phosphatases play an important role in mediating such signal transduction pathways.

A cell includes many nano-machines, which work in coordinated and concerted manner to carry out innumerable important functions. For instance, the important criterion for sustenance involves the basic processes such as DNA replication, transcription, translation followed by various Post-Translational Modifications (PTMs). PTMs are central to protein structure regulation and function which in turn determine and modulate protein catalytic activity, conformation, sub-cellular localization, stability and interaction with other proteins [1]. A number of PTMs occur inside the cell such as phosphorylation, glycosylation, acetylation, thiolation, adenylation, ribosylation, ubiquitination, myristoylation and palmitoylation among others [2], however, the profound outcomes of phosphorylation events are considered to be of higher significance. Phosphorylation events are commonly observed in both prokaryotes and eukaryotes. It is estimated that about one-third of eukaryotic proteins are phosphorylated [3, 4]. In-depth genome sequence analysis of *Arabidopsis thaliana* has revealed that it has more than 800 protein kinases [5] and nearly 150 protein phosphatases [6]. Phosphorylation mediated functional modulation of the target protein includes alteration of enzyme activity, cellular localization, or association with other proteins. Protein kinases occur ubiquitously across bacteria, plants and animals kingdom. The protein kinases act on the substrate by transferring a phosphate group and covalently attaching it to a particular amino acid side chain with a free hydroxyl group. Usually a nucleotide triphosphate such as ATP serves as phosphate group donor.

The protein kinases and phosphatases have been classified based on the type of amino acids they target for phosphorylation and de-phosphorylation respectively [7]. In prokaryotes, histidine, aspartic acid and glutamic acid are the preferred targets of kinases for phosphorylation, whereas in eukaryotes serine, threonine and tyrosine phosphorylation is more frequent. Although serine and threonine residues also undergo phosphorylation in bacteria, but their kinases substantially differ from that of eukaryotes. A substantial num-

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ber of eukaryotic kinases target both serine and threonine amino acids for phosphorylation hence called as serine/threonine kinases, while some others target tyrosine. Besides these, a few kinases are termed as dual specificity kinases as they target both serine/threonine and tyrosine residues [8]. Certain kinases such as histidine and aspartate that are less in number phosphorylate histidine and aspartate residues.

Another major group of counter-regulatory proteins to kinases called as phosphatases exist in the cells, which catalyze the removal of phosphate group from proteins. The dephosphorylating activity of phosphatases is quite specific. As mentioned above, kinases and phosphatases are the key components of signal transduction machinery acting as molecular switch for specific pathway and hence regulating the desired biological process. In order to decipher the signaling cascades mediated by phosphorylation and dephosphorylation events, several effective tools and techniques have been developed. These methods aid in substrates identification for kinases, essential to reveal the underlying processes that can further help in mechanistic understanding of signaling pathways.

The phosphorylation/dephosphorylation of proteins can have profound effects on protein structure, activity, subcellular localization and interaction with other biomolecules [9-11]. Phosphorylation also results in the formation of binding sites for specific proteins or biomolecules to initiate a cellular response as per the cell's need [11]. It has been reported that about 2-4% of functional eukaryotic genes encode for protein kinases, [12] which might phosphorylate 30% of the proteome [13] suggesting their role in many regulatory and metabolic processes. Moreover, in plants, protein phosphorylation has implications in various biotic and abiotic stress responses, which further leads to signaling cascades *via* range of stimulus such as pathogens, light, hormones and temperature. In order to determine kinase activity towards substrates, researchers have been using many different techniques with limited success (Fig. **1**).

The interaction between kinases and substrates is transient in nature. The activity of most of the kinases is of ephemeral nature and due to this, despite the current technical advancements; we do not yet have a solution in the form of an efficient technique to pull out a protein of interest from a pool of diverse proteins. Herein, we are elaborating on the available tools and techniques to identify the targets/substrates of kinases with their advantages and limitations.

## 2. TECHNIQUES FOR ELUCIDATION OF SUBSTRATES FOR KINASES

The need for identification of substrates for protein kinases to deduce the signaling pathways prompted the advent of new technologies. The identification and characterization of kinase substrates are usually initiated with simple experiments such as Protein-Protein Interaction (PPI) analysis. Low throughput approaches of PPI include biophysical methods like Nuclear Magnetic Resonance (NMR), crystallography and an array of spectroscopic and calorimetric methods. Although these methods hold a vital place in conducting such studies, more recently a number of high throughput approaches have been adopted to gain a new insight into PPI on a large scale.

### 2.1. Two-hybrid Screening

Traditionally, for comprehensive study of PPI, two high throughput methods are extensively used. These methods are yeast two-hybrid screening and Tandem Affinity Purification (TAP) tagging. The two-hybrid screening became popular owing to its simplicity and promptness in finding the interacting partners of a target protein [14, 15]. Particularly, this method is helpful in finding proteins that interact as a binary complex with the target protein. The screening method uses a reporter gene such as *E. coli lacZ* gene. The activation and expression of *lacZ* gene is readily detected by the blue coloration of the transformed cells when grown in a medium containing X-Gal (5-bromo-4-chloro3-indolyl-β-D-galactopyranoside) [15]. The gene is activated only when an adjacent region of DNA called as upstream activation sequence (UAS) associates to a protein containing a transcription activation domain. Thus, interaction of DNA binding sequence and transcription activation sequence results into the formation of functional β-galactosidase enzyme (*lacZ* gene product) followed by X-Gal breaking down to yield galactose and 5-bromo-4-chloro-3-hydroxyindol. The dimerization and oxidation of 5-bromo-4-chloro-3-hydroxyindol into 5, 5'-dibromo-4, 4'-dichloro-indigo yields an insoluble blue product, which is easily detectable [15]. This methodology utilizes the molecular architecture of the transcription factor harboring two domains *viz*. DNA binding and the transcription activation domain facilitating the PPI by splitting them into two parts. During screening, the DNA binding domain is fused to a putative dimerization domain, whereas the transcription activation domain is fused with different clones to a library of putative dimerization partners. The reporter gene activation is detected only when the two-dimerization domains are in proximity and they interact with each other. This results into binding of transcription factor onto the UAS followed by transcription of the reporter gene, giving rise to blue colonies.

### 2.2. Tandem Affinity Purification (TAP)

Imperfection issues that are generally encountered during immunoprecipitation can be sorted out by using Tandem Affinity Purification (TAP) [16]. This technique is based on the usage of dual affinity tagged-fusion proteins provided with a strong antigenic region (*e.g.*, Protein G) joined in tandem with a separate tag, such as streptavidin. Generally, the two tags are kept apart by a specific exogenous cleavage site, usually the Tobacco Etch Virus (TEV) protease. The TEV protease cleaves a sequence (Glu-X-X-Tyr-X-Gln/Ser), which is not very common in mammalian proteins thus minimizing the risk of cleaving the bait protein and/or associated protein. After induction and expression of TAP-tagged bait, protein complexes are separated by two different affinity purifications. The linkers and small peptide tags, which are used in the second purification step enable specific elutions thus, enhancing the specificity of pull-downs [17]. Although TAP-tagging has been used in many fields, its application for large-scale interactome analysis is time consuming and highly priced. Moreover, as the whole process of purification includes many

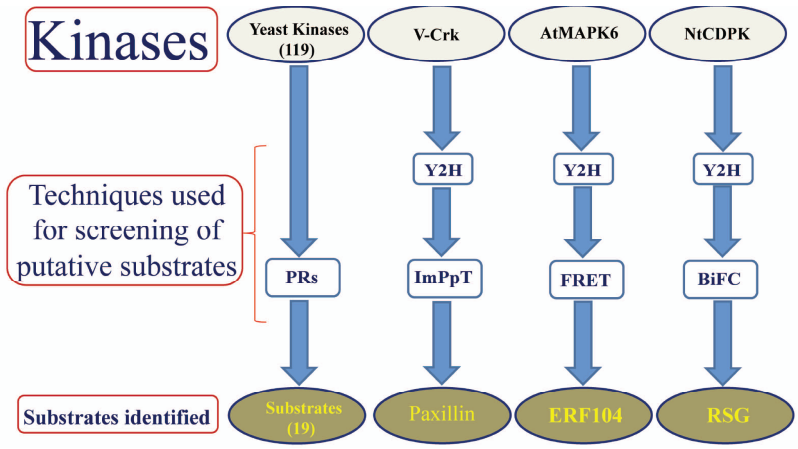


**Fig. (1).** Substrates of kinases screened and validated by Y2H (yeast two-hybrid), PRs (protein microarrays), ImPpT (immunoprecipitation), FRET (Fluorescence Resonance Energy Transfer) and BiFC (Bimolecular fluorescence complementation assay). The green oval shows the kinases, rectangular boxes indicate techniques used for screening of putative substrates followed by its further confirmation while pink oval represents substrates identified. Zhu *et al.* in 2001[85] used Protein microarray for the identification of substrates for yeast kinases. Immunoprecipitation technique helped to validate interaction of v-Crk SH2 domain and demonstration of paxillin phosphorylation by GST fusion immuno-precipitation [29]. FRET aided in reporting the *in-vivo* interactions of the MAPK from *Arabidopsis thaliana* - MPK6 with an Ethylene Responsive Factor104 (ERF104). Identification of putative substrate (transcription factor) of MPK6 by screening through yeast-two hybrid, followed by validation of interaction *in-vivo* by FRET [48]. Ito and group confirmed the interaction of NtCDPK with RSG (REPRESSION OF SHOOT GROWTH- a transcription factor involved in gibberellin homeostasis) by BiFC by fusing RSG and NtCDPK with YFPn and YFPc, respectively thus confirming their interaction. (*For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.*)

steps of purification, weaker or transient binding partners may not be detected in the final eluate.

### 2.3. *In Vitro* Identification of Potential Substrates of Kinases

In the past few years, combined efforts toward development of systems for the identification of protein kinase substrates have led to the birth of certain *in-vitro* techniques. However, in spite of their utility, they sometimes yield misleading results and thus require further validation using other *in-vivo* techniques. The *in-vitro* kinase assay although convenient, has certain limitations associated with it. It has been shown that certain physiological functions such as regulatory mechanisms of kinases are disturbed upon purification from the cell [18, 19]. This problem can only be solved with sufficient kinase regulatory information coupled with efficient purification method. Fukunaga *et al.* (1997) have developed a novel technique known as phosphorylation screening for the substrate identification [20]. It involves the usage of purified protein as well as phage cDNA expression library plated on nitrocellulose membrane along with the desired purified kinase and radioactively labeled [$\gamma$-$^{32}$P]-ATP. For detection, autoradiography is performed in order to identify the desired phage and their substrates. Sequencing the cDNA to reveal identity of the substrate does further confirmation.

The method involves construction of a λGEX5, a GST expression vector for its utilization in identification of positive cDNA clones that can be further used for expression and purification. Screening of clones from HeLa cDNA library for ERK, MAPK substrates by the same group further proved the effectiveness of this method. Additionally, the comparison of phosphorylation status of already existing substrates p90$^{RSK2}$ and c-Myc by MAPK using this method has strengthened its inference [21, 22]. Apart from the above-mentioned kinases, substrates for ERK2MAPK [23], cyclinE/Cdk2 [24], Akt/PKB [25] and PAK1 [26] were also identified using this method. This technique can thus, potentially be employed for the target identification of different kinases. Moreover, the underlying principle of solid phase phosphorylation is in line with the conventional liquid-phase phosphorylation since proteins are immobilized on a membrane filter in both of them.

Besides the identification of phosphorylation specificity, peptide specificity also plays significant role. Apart from this there are other factors for determining substrates such as substrate recruitment, which involves the proximity and association of kinase with the putative substrate for formation of strong enzyme-substrate complexes. During such recruitment, substrate binding is observed to occur at different kinase domains such as regulatory domain, sites other than the active site or the catalytic site [27]. Auto-phosphorylation also helps in recruitment of the substrate as both substrate and kinase are in close proximity to each other [28].

## 3. IMMUNOPRECIPITATION

This technique comprises purification of a protein complex immobilized by an antibody against a known component. The basic steps involved are preparation of cell lysates and addition of specific antibody followed by antigen precipitation and washing. The antibody can be expressed in suitable hosts and purified. Alternatively, epitope tags such as GST and His, if bound to the protein can also be utilized, as glutathione or Ni conjugated beads are available commercially, which can help in purification of the protein. For example, interaction of v-Crk SH2 domain and phosphorylated paxillin was demonstrated by GST fusion immunoprecipitation [29]. The bound protein complex obtained is further eluted and analyzed. This method has been applied in various experiments and is considered to be a classical approach for the identification of PPI. Unlike yeast-two-hybrid

(Y2H) or Mass Spectrometry (MS), this method cannot be applied in a high-throughput manner but is advantageous for the analysis of endogenous proteins [30]. Immunoprecipitation was employed to isolate phosphorylated proteins using an antibody specific for phospho-motif generated by the kinase. For instance, 160-kDa substrate of the protein kinase Akt– a serine/threonine kinase, which phosphorylates RXRXXS/T motif, was identified using immunoprecipitation approach. Further confirmation of the phosphorylation sites was done using MS and site-directed mutagenesis [31].

Various players are involved in perception and signal transduction of hormones in a signaling cascade. One of the largely studied phytohormones, Brassinosteriod (BR) regulates various aspects of plant growth and development. BR signal transduction involves activation of two receptors BRASSINOSTEROID-INSENSITIVE (BRI1) and BRI1-ASSOCIATED RECEPTOR KINASE1 (BAK1) by BR. For depicting *in-vivo* association of BRI1 and BAK1, co-immuno-precipitation assay was used. It was reported that phosphorylation of both, BRI1 and BAK1 on threonine residues was BR dependent. *In vivo* phosphorylation sites of BRI1 were identified by immuno-precipitation of epitope tagged BRI1 from *Arabidopsis thaliana* followed by liquid chromatography and tandem MS [32]. The limitations (presented in Table **1**) can be prevented to certain extent by precipitating the proteins using only specific and not cross-reacting antibodies. Additionally, monoclonal antibodies can be used for more specificity [29]. Another essential point to be considered is the non-specific covalent binding of kinase to agarose beads, IgG molecules *etc*. It may lead to pull down of non-specific kinases. This was found to be more prominent in the case of biological materials containing secondary metabolites such as phenolics and in samples that are harvested after stress treatment [33]. Although the identification of non-specific kinases can easily be determined by difference in size to that of the kinase of interest [34], other important criterion that must be taken into account is buffer composition (concentration of salts and detergents added). The major drawback of this technique is that it cannot be applied for large-scale phospho-proteomic studies [35]. However, the most remarkable advantage of immunoprecipitation is the sensitivity provided by the use of antibody.

## 4. FRET AND BiFC- IMAGING TECHNIQUES

The advent of new technologies and the reformations in microscopy have paved the way to study different molecular interactions occurring inside the cell. Beginning from the light microscopy to advance confocal, Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM) have enabled elucidation of the different facets of functional and structural aspects of a typical cell. The study of various PPI occurring inside the cell helps in understanding the networking of various proteins in signaling cascades and consequently for re-programming of cellular machinery. The daunting task of identification of substrates for kinases is further eased by application of certain *in-vitro* techniques such as Bimolecular Fluorescence Complementation (BiFC) and Förster Resonance Energy Transfer/Fluorescence Resonance Energy Transfer (FRET). These can be efficiently used for PPI analysis and hence in understanding possible implications of such interactions.

The spatial and temporal regulation of kinases inside the cell can help in decoding the signaling cascades. This can be possible by their investigation in living cells. The discovery of Green Fluorescent Protein (GFP) from bioluminescent jellyfish *Aequorea victoria* in 1992 revolutionized the field of imaging in biology for observing the signals *via* live imaging [36]. GFP protein is excited by ultra violet (UV)-light (395-470 nm) and the three amino acids located in the core of the GFP protein namely serine-tyrosine-glycine are oxidized and make p-hydroxybenzylidene-imidazolone chromophore, which emits green light with a wavelength of 509 nm [36]. For assessing the interaction between a kinase and a putative substrate, FRET can be utilized where the transfer of energy between fluorescently labeled proteins of interest and phospho-specific antibody conjugated with acceptor fluorophore takes place. Different variants of fluorescent protein tags such as Green Fluorescent Protein (GFP), Cyan Fluorescent Protein (CFP), Yellow Fluorescent Protein (YFP) and Red Fluorescent Protein (RFP) are now widely used in tracing the subcellular events. Hence, the natural rainbow-like phenomenon can aid in understanding the language of signaling using FRET.

### 4.1. Förster/Fluorescence Resonance Energy Transfer (FRET)

FRET involves two fluorophore proteins where non-radiating transfer of energy from donor to acceptor fluorophore proteins results into fluorescence emission from the acceptor fluorophore. Appropriate distance and orientation of the fluorophores are vital for the FRET efficiency [37]. The visible fluorescence proteins are not only required to study localization patterns but they also elucidate various molecular interactions occurring inside the cells. Spectral overlap of acceptor and donor, the distance between the two molecules and the relative orientation of the two-chromophore dipoles are some of the prerequisites for this technique [37]. FRET efficiency is inversely proportional to the sixth power of the distance between the two flourophores. Using this property, Stryer in 1978 considered FRET as a spectroscopic ruler to measure the distance between two sites on macromolecules [38]. Quantification of FRET efficiency is usually done by two methods- the first method involves measurement of fluorescence signals from donor, acceptor or both using three filter sets [39] and the other is a simple approach measuring de-quenching of the donor after acceptor bleaching [40]. Longer wavelengths (>500nm) leading to strong illumination bleaches the YFP without affecting CFP thus, optically eliminating YFP [41, 42]. De-quenching of the donor after photobleaching is a damaging process but best explains the specific, definite and accurate interactions [37]. Hence, the overlapping absorption and emission spectrum between two identical fluorophores lead to FRET. Now-a-days CFP and YFP are considered as the most suitable FRET fluorophore pairs. Some of the other popular FRET pairs being used are Green/Red (EGFP/mCherry) [43, 44], Cyan/Orange (MiCy/mKO) [45] and Yellow/Red (mAmetrine/tdTomato) [46]. The methodology of FRET has been utilized to detect the phosphorylation reaction by kinases on their substrates where phosphorylation between two fluorophore proteins

**Table 1. List of the different techniques along with Pros and Cons discussed in the review.**

| Techniques | Pros | Cons |
|---|---|---|
| **Two-hybrid screening** | Simple, rapid and cheap method to find the interacting partners | Gives false positive results |
| **Solid phase phosphorylation assay** | Enables detection of even less abundant proteins<br><br>Transient complexes of kinase-substrate are highly recognizable by this technique<br><br>Screening is specific only for the identification of substrate proteins<br><br>Protein induction and expression is much easier as the GST-fusion protein tag is attached to the cDNA<br><br>Has been proven to be better than peptide library screening | Occurrence of incorrect folding of the expressed proteins<br><br>Absence of ambient conditions which prevail *in-vivo*<br><br>Differential localization of both- kinase and the substrate leads to slippage of phosphorylation sites<br><br>Less active and specific enzymatic performance by kinases *in-vitro* conditions<br><br>Possible kinase phosphorylation of unwanted or non-specific proteins derived from *E.coli* might take place which creates difficulty in distinguishing positive plaques from the negative ones<br><br>High amount (micrograms) of active and soluble purified kinase is required<br><br>Another possibility of obtaining false positive is by auto-phosphorylation of kinases or other non-specific proteins in its immediate vicinity<br><br>Presence of protein kinase of low substrate specificity may lead to interaction with non-physiological substrates than the target ones |
| **Immunoprecipitation** | Advantageous in analysis of endogenous proteins<br><br>The most remarkable advantage of immunoprecipitation includes sensitivity provided by antibody | Unlike Yeast-two-hybrid (Y2H) or Mass Spectrometry (MS), this technique cannot be applied for large-scale phospho-proteomic studies |
| **Förster/Fluorescence Resonance Energy Transfer (FRET)** | Use of fluorescent biosensors give high sensitivity and selectivity<br><br>Does not damage the tissue and the biomolecules of interest<br><br>Helps in studying *in vivo* interactions allowing live cell imaging<br><br>Helps in scrutinizing the internal physiological processes<br>Helps in localization in organelles | Auto-fluorescence of the cells creating a background noise<br><br>The donor reabsorbs the emission |
| **Bimolecular-Fluorescence Complementation Assay (BiFC)** | A simple and easy to handle technique without any requirement of external reagent<br><br>Absence of background noise as only reconstitution of the two co-expressed entities leads to expression of YFP<br><br>The sub-cellular localization of the two interacting proteins is revealed *in-vivo*<br><br>The transient and weak interactions are also easily established by this sensitive technique<br><br>Direct visualization is possible due to strong intrinsic fluorescence | As compared to the real time protein-protein interactions, the slow maturation time of reconstituted GFP/YFP/CFP restricts the timely detection of dynamic changes occurring over short span of time<br><br>The analysis of protein-protein dissociation is impeded by stability of the tethered YFP moieties. And this may lead to detection of non-specific signals because the YFP fragments occurrence frequency is very high<br><br>Unlike the native proteins, the amalgamated proteins may have different properties and might provide spurious results |

**(Table 1) contd….**

| Techniques | Pros | Cons |
|---|---|---|
| **Mass Spectrometry** | A useful tool for elucidating the phosphorylation sites of proteins<br><br>High throughput nature of MS helps in large-scale identification of phosphorylation sites | |
| **Protein arrays** | The conditions under which protein microarrays operate can be modulated up to a certain extent. These modifications may be change in pH, temperature and ionic strength<br><br>Significant due to its ability to assess a large number of proteins at the same time with least amount of sample.<br><br>Helps in the differential utilization of protein samples by fabricating them in parallel with different probe molecules under different conditions | Lacks specificity and magnitude of occurrence<br>Another major concern is the cross-reactivity with antigen of no relevance, thereby affecting the available amount for the protein of interest<br>Lack of suitable information about the proteins and their respective affinity reagents<br>Inability to identify less abundant proteins |

results into a change in conformation that can be utilized for creating FRET-based kinase sensors [47]. Live imaging or visualization can thus be made possible with the help of these genetically encoded biosensors. Generally, the parallel orientation and short distance ensure effective FRET. Bethke *et al.* in 2009 have reported *in-vivo* interaction of the MAPK from *Arabidopsis thaliana* - MPK6 with an Ethylene Responsive Factor 104 (ERF104) using FRET. They first identified the putative substrate (here a transcription factor) of MPK6 through yeast two-hybrid screening, followed by validation of interaction *in-vivo* by FRET [48]. This resulted in confirmation of ERF104 as a nuclear localized substrate of MPK6. The MPK6-ERF104 complex formation was rapidly lost in the presence of flagellin-derived flg22 peptide. The flg22 peptide mediated loss of MPK6-ERF104 interaction requires not only MPK6 activity but also ethylene signaling. The involvement of ethylene signaling hints toward a novel role of ethylene in substrate release, supposedly allowing the liberated ERF104 to access target genes [48]. Another report from Russinova *et al.* in 2004 put forward a study on Leucine Rich Repeats (LRRs)-like kinase that was considered to be involved in BR perception. They performed *in-vivo* studies by fusing BRI1 and BAK1 to cyan and yellow fluorescent variants. These fused proteins were then transiently expressed in cowpea protoplast and their FRET localization was observed in endosomes and plasma membrane suggesting that BAK1 may help in regulating endocytosis of BRI1 [49].

### 4.2. Bimolecular-Fluorescence Complementation Assay (BiFC)

Another widely used microscopic method to reveal the PPI or the kinase-substrate interaction is Bimolecular Fluorescence Complementation Assay (BiFC). In addition to various techniques dedicated to determine stable interactions between proteins of interest, there is a need of a method that can be utilized for the identification of transient interactions existing between enzyme-substrate complexes. BiFC primarily functions on a similar principle as split-ubiquitin system of yeast two-hybrid. For this reason, it is alternatively known as split-YFP assay. The key to this process includes usage of half or split fluorescent protein where both the halves are bound to the proteins of interest to be examined for interaction. The different fluorophores used include CFP, YFP and RFP. As a consequence of the interaction between the proteins of interest, reconstitution of the non-fluorescent ends *i.e.* the N- and C-terminals leads to the formation of a fully functional fluorescent protein [50]. Thereby, providing an easy procedure where no external reagent or source is required to visualize the interaction.

It was in 2002 that Kerppola and co-workers came up with this unique concept for unveiling the protein interactions. Since then, the application of BiFC has widely spread from animal or cell cultures [51] to plant systems such as *Arabidopsis* [52, 53].

### 4.3. Evidence from Plant Systems

The extensive usage of BiFC in *Arabidopsis* and other plants is credited to Bracha-Drori *et al.* (2004) and Walter *et al.* (2004). Some of the recent reports including one by Pusch *et al.* (2012) highlighted the application of BiFC for identifying the substrate of a major cell cycle kinase in *Arabidopsis* [54]. They first screened the substrates of CDKA (A-type cyclin-dependent kinase, a homologue of cdc2) using the split-ubiquitin system with certain interactors such as CKS (A tyrosine-protein kinase; also known as C-Src kinase or C-terminus Src kinase), CYCB1; 2 and CYCD3; 1 and later on substrates like CDC6, CDT1 and histone H1, which were further validated by BiFC. They analyzed the co-infiltrated leaves of tobacco (*Nicotiana benthamiana*) with expression vector for CDKA1, which yielded prominent YFP signals upon interaction with the corresponding interactors. This provided strong evidence

that substrate-kinase interaction identification can be supported by BiFC technique [54]. Similarly in 2010, Ito and group confirmed the interaction of NtCDPK with RSG-(REPRESSION OF SHOOT GROWTH) a transcription factor involved in gibberellin homeostasis when RSG was fused with YFPc and NtCDPK with YFPn to confirm their interaction [55]. Another group in 2009 also confirmed the rice kinase-protein map by BiFC after screening about 116 rice kinases leading to 378 kinase-protein interactions *via* yeast two-hybrid system [56]. They used 6 kinase-protein interaction pairs by fusing protein downstream of the YFP construct and then transiently expressing them in etiolated protoplasts derived from rice seedling [57] and detected YFP signals from four out of the six pairs used [58].

The most appreciable application of this technique is that it enables easy detection of even transient and weak interactions [59]. Direct visualization is possible due to strong intrinsic fluorescence [60]. Despite being a powerful technique, it has certain disadvantages such as the slow maturation time of reconstituted GFP/YFP/CFP and this restricts the timely detection of dynamic changes occurring in a cell over a short span of time [59, 61, 62]. The introduction of Venus variant of YFP in the following years provided the solution to this problem [37]. The analysis of protein-protein dissociation is impeded by stability of the tethered YFP moieties. This may lead to detection of non-specific signals because the YFP reconstitution occurrence frequency is very high. Unlike the native proteins, the amalgamated proteins may have different properties and might provide spurious results [63].

### 4.4. Possible Improvement

Recent progress in the BiFC involves the usage of multicolor fluorophore proteins together in order to study the multiple interactions taking place at a given time within the cell. This will further help in determining the different interactomes of the signaling pathway [64]. Thus, multicolor complementation would be of great use to understand comprehensively the signaling networks but require meticulous exercise to create such oligomeric probes and biosensors.

### 4.5. Race Between FRET and BiFC - Who Wins?

Both of these visualization techniques are widely used for studying the putative interaction between kinase and their respective substrates. But neither of the two can be regarded as perfect because of certain flaws associated with each of them. FRET requires chemically bound or genetically fused two fluorophores whereas BiFC has the fragment complementation. The level of protein expressed is of great importance in case of FRET, as it requires good amount of protein for detection. Besides this, the distance between the two fluorophores plays a pivotal role in their detection because a distance greater than 100 Å will not lead to energy transfer. Like any experimental set-up this also requires proper controls in order to nullify any sort of false positive results. Regardless of all these fallacies, FRET has still contributed greatly to a wide number of protein interactions. But it cannot be denied that as compared to the BiFC, it can help in detection of duration of complex formation as well as their dissociation [60].

Moving on to BiFC, its applicability in revealing the interaction between proteins makes it one of the most widely used techniques. The greater extent of applicability was achieved due to its diverse characteristics such as intrinsic property of fluorescence upon fragment reconstitution; ability to present clear view due to absence of tampered cells caused due to cell lysis. It also helps in estimating the endogenous protein level, as the amount of protein used for analysis is relatively similar to that present inside the cell. This method can also be beneficial when multicolor fragments are used in order to study the existing discrete set of proteins present inside any living cell. To sum up, BiFC does not require any further interpretation of the data as it gives a direct evidence for the possible interaction. Hence, BiFC is emerging as an influential visualization technique [60].

## 5. HIGH-THROUGHPUT APPROACHES

### 5.1. Mass Spectrometry

Mass spectrometry is a sensitive high-throughput screening tool, which is an improvement over the traditional biochemical technique such as Edman degradation. It is a useful tool for elucidating the phosphorylation sites of a protein. This has helped in identification of many novel phosphorylation sites. The process involves a two-step approach including digestion of the phosphoprotein of interest by trypsin enzyme (trypsin digestion) and then the peptide units are analyzed for determining the phosphorylated sites. This is followed by tandem mass spectrometry (MS/MS), which helps in determining the exact location of the particular amino acid site being phosphorylated [65]. The identification of the phosphopeptides becomes easier as the addition of the phosphate group leads to an increase in protein mass of about 80 Dalton [66].

The success of the technique depends on the kind of sample prepared as well as the type of mass spectrometer used. Basically, two methods are utilized for the MS namely, matrix assisted laser desorption/ionization (MALDI) and Electrospray Ionization (ESI) that may be used separately or when, used in combination yields better results. Both of these approaches help in attaining the mass for the target phosphoprotein [65]. The high throughput nature of MS helps in large-scale identification of phosphorylation sites [66]. The main precaution that must be taken into consideration involves the inhibition of activity of phosphatases that could dephosphorylate the sites during cell lysate preparation and purification [67]. As there are different kinds of residues that are being phosphorylated namely serine, threonine and tyrosine in eukaryotes, there are many techniques that are used in combination with MS for phospho-enrichment. To state, some of them are Immobilized Metal Affinity Chromatography (IMAC) and chemical modifications.

### 5.2. IMAC

Immobilized Metal Affinity Chromatography utilizes metal chelation as phenomena for enrichment of phosphopeptides. Here, metals such as $Fe^{3+}$ [68], $Ga^{3+}$ [69], $Zr^{4+}$ *etc*. are used for enriching highly negative phosphate groups toward themselves. This technique helps in better enrichment of serine, threonine and tyrosine residues [70] as compared to acidic groups such as glutamic acid and aspartic acid. But

this constrain was removed by modifying the acidic residues by esterification [71]. The type of metal ion used, column material and elution procedure play important roles in recovering good quality phosphopeptides.

### 5.3. Chemical Modifications

This technique is also beneficial for the enrichment of phosphorylated residues. This includes changing of the chemical environment such as acidic to alkaline. On exposure to alkaline conditions [72], the phosphorylated residues undergo β-elimination leading to the detection of dehydroalanine or dehydroamiobutyric acid with the help of tandem mass spectrometry (MS/MS) [73]. The first major breakthrough was the report based on the study of RLKs (Receptor-like kinases) that are phosphorylated inside as well as outside their kinase domains [74]. In *Arabidopsis*, substrates for BRASSINOSTEROID INSENSITIVE1 receptor kinase (BRI1) kinases were identified by revealing about 11 phosphorylation sites using a combination of LC/MS/MS and IMAC [75].

These enrichment techniques along with MS serve as a better tool for identification of phosphopeptides as well as give lower noise of non-phosphorylated peptides, thereby increasing the sensitivity for selection.

### 5.4. Protein Arrays

Proteins majorly account for the well-being of biological systems thus playing an important role in almost each and every aspect of the different physiological pathways inside the cell. The development of different techniques for their quantification as well as characterizing their functions has emerged during the past decades. One of them includes protein arrays or microarrays. Microarrays commonly known as lab-on-chip, comprise of patterned arrangement of biomolecules such as DNA or protein on a solid support such as glass slide or plate. The origin of microarray was from a technique established in 1989 by Roger Ekins [76] known as ambient analyte immunoassay. The first known use of microarray was reported in 1995 [77]. With the introductory success of DNA microarrays for studying the genomic and RNA profiles, focus shifted toward the proteomics profiling. Thus, giving a new hope for better understanding of the proteome. Chen and Zhu (2006) classified arrays as functional and analytical arrays. Analytical arrays comprised of the antibody-antigen recognition concept whereas functional protein microarray takes into account the characterization of proteins from an organism [78]. Moreover, recently another microarray modification utilizing cell/tissue lysates known as reverse phase array has come to the fore as an alternative to the analytical arrays [79].

### 5.5. Analytical Protein Microarray

It is considered to be the armature for identification of proteins using antibody for detection purposes. However, it has certain limitation such as lack of specific protein labeling and identification of less abundant proteins [80]. In order to achieve more sensitivity, it can be modulated like the sandwich ELISA, which involves the participation of two antibodies *i.e*. capture and detection antibodies [79, 81]. This thereby, increases the sensitivity and unambiguous criteria for selection [78, 79]. In spite of using antibodies for detection in case of protein microarrays, it has certain drawbacks such as specificity and magnitude of occurrence [82]. Creation of specific antibody is a cumbersome task but the usage of recombinant antibodies in order to prevail over limitations such as non-specific binding [83]. Another major concern is the cross-reactivity with antigen, thereby affecting the available amount of protein of interest.

### 5.6. Functional Protein Microarrays

Employment of functional protein microarrays for evaluating the cellular proteins is a routine technique. This also helps in elucidating various biochemical properties of proteins such as PPI, protein-peptide interaction and enzyme-substrate association [78, 79]. The generalized procedure involves the application of purified protein samples in the form of separate spots over glass slide. The protein purity and its integrity are very important for functional protein microarrays. Thus, protein microarrays represent a high-throughput approach [84]. This does not require denaturation of the proteins. The most commonly used surfaces are the chips and slides. In order to prevent denaturation of the proteins and reduce evaporation, modification of the surface is usually done and this may include nano-wells or microfluidic channels. This modification in turn leads to increased binding affinity of the protein. In 2001, Zhu *et al.* (2001) first reported its use in identification of substrates for yeast kinases. They targeted 122 protein kinases in yeast for substrate identification. Albeit out of 122, they could only express and purify 119 kinases. Using these, they proceeded for substrate combination utilizing about 16 peptides. They finally used 17 substrates for 119 kinases [85]. This turned out to be a starting point for studying cellular proteome allowing its application into various organisms such as humans, yeast and plants [78, 86, 87].

### 5.7. Reverse-Phase Protein Microarray

As the name suggests, it operates in reverse manner to the basic arrays. It directly employs application of sample in the form of cell lysates/fractions on the glass slide. In 2001 Paweletz and co-workers first reported its use in histological studies of prostate cancer [88]. However, due to its dependence on specificity of antibodies and their availability, it has limited application [80]. The study of yeast kinome has helped in identification of kinase substrates as well as new tyrosine phosphorylation activity [89]. Once again using yeast kinome, Snyder's group carried out a large scale "phosphorylome project". They individually incubated about eighty-seven purified kinases on yeast proteome array using appropriate kinase buffer along with labeled $^{33}$P-$\gamma$ -ATP. As a result, a total of about 4129 phosphorylation sites were identified in conjunction with 3125 substrates [89]. Progress has been made from the kinome studies of unicellular organisms toward the multicellular primary producers. Starting from the model plant to cereals, kinome studies have been done in order to elucidate the downstream target of the signaling cascades. As in the case of other techniques, this system also has certain pros and cons. The cons include lack of suitable information about the proteins and their respective affinity reagents. Another important point is its inability to identify less abundant proteins, as they might not be able to

build sufficient competition with the existing abundant proteins. The positive aspects of array techniques are the conditions which can be modulated to some extent. These modifications can be either change in pH, temperature and ionic strength.

The microarrays are significant due to their ability to assess large number of proteins at the same time with least amount of test sample. In addition, the differential utilization of protein samples by fabricating them in parallel with different probe molecules under different conditions favors its comprehensive usage. Thus, microarrays can be considered as a perfect technique for system-oriented proteomics.

### 5.8. Evidence of Protein Microarray in Plants

Apart from its utilization in human and yeast systems for deciphering the PPI, kinase-substrate complex formation, post-translational modifications [90], the protein microarray is extensively applied in plant system as well. In 2007, Popescu and co-workers used this high-throughput technique to decipher the function of model plant protein. They created a high quality expression library for protein samples of Calmodulins (CaMs) and Calmodulin-Like Proteins (CMLs), as they constitute a major part of plant proteins. About 1133 purified proteins were taken into account and different CaM/CML were used for probing, namely 3 CaM (CaM1, CaM6 and CaM7) and 4 CMLs (CML8, CML9, CML10 and CML12). As a result, an assorted pool of 173 putative substrates was identified [91].

### 5.9. Bioinformatics Approach for the Identification of Substrates of Kinases

Bioinformatics has played a worth-mentioning role in the identification of biological components and targets of cellular pathways by a precise algorithm based computation and prediction exploiting large *in-silico* resources or databases. Although there are different techniques that are being utilized for the purpose of identification of substrate for kinases or phosphoproteomics but bioinformatics has entered the ocean like a bloom. The different type of modifications of *in-vitro* and *in-vivo* methodologies are currently used but with limited success owing to constraints such as time consumption, cost, availability of enzymes for reactions, machinery and infrastructure. *In-silico* methods can play a pivotal role in overcoming these drawbacks. The *in-silico* computational methods can provide simpler approach for elucidation of phosphoproteome profile. The prediction systems perform well when a large number of kinase information is provided. By rigorous machine training and development of logical algorithm, bioinformatic analysis has emerged as a tool of choice for prediction of substrate of a kinase responsible for phosphorylating a particular site of the respective substrate. There are recent advances observed in this field too. Various algorithms used for prediction provide a statistically significant indication of putative interaction that can be further confirmed by using different *in-vitro* techniques. Since none of the system software is accurate and almost always accompanies false virtues; efforts are being made to improve the prediction tools to remove these shortcomings. There are different tools specific for model plant *Arabidopsis* such as PhosPhAt [92, 93], PlantPhos [94]. However, for other plant systems identification is still a tough task, as databases are not yet developed for them. Recently, SAPHIRE (SAskatchewan PHosphorylation Internet REsource), a new prediction tool has been specifically developed for plants using soybean as a system [95]. Algorithms based on different parameters that are considered for identification of phosphorylation sites are employed by these databases. Some of the sets on which these prediction tools work include Position-Specific Scoring Matrices (PSSMs), Support Vector Machines (SVMs), genetic algorithms, Artificial Neural Networks (ANNs), decision trees *etc*. Thus, it would not be significant to compare different databases for identification as they work in a discrete manner [96]. Amongst the above-mentioned sets, PSSM is the easy to use technique, which is represented in the form of a matrix, where rows and columns demarcate amino acids and multiple sequence alignment, respectively. The matrix depicts the occurrence of frequency of a particular amino acid in a given position. This technique does not help in recognition of patterns that involve the amalgamation of amino acids as a crucial phenomenon. But this gap is generally filled by the utilization of other methods such as ANNs and SVMs [97]. The number of residues in the proximity of phosphorylation sites defines the criteria for the phoshorylation prediction. Some tools take into account few residues that lead to overlooking the prediction, while some consider too many residues that reduce the background noise. Some *in-silico* methods are also used for prediction of the 3-D structure of the phosphorylation sites [98] such as DISPHOS and Netphos K. The tools involved in the identification of the various putative phosphorylation sites have allowed the development of the certain databases, which can be utilized for this purpose as mentioned in Table **2**.

## 6. SIGNIFICANCE OF PROTEIN PHOSPHORYLATION DURING STRESS CONDITIONS IN PLANTS

As mentioned earlier, protein phosphorylation is the most significant type of PTMs in the life cycle of all cells. Reports suggest that at any given time approximately one-third of all proteins are phosphorylated on Tyr, Ser or Thr residues in eukaryotes [114, 115]. Here, in this section, we briefly summarize the phosphoproteomics and the impact of protein phosphorylation in combating various stress conditions.

### 6.1. Salinity

About 20% of the arable and nearly half of the total irrigated land of the world are affected by salinity [116, 117]. High concentration of salt in the soil and water used for irrigation impose threat to optimum plant metabolism and can interfere with cellular homeostasis [118]. The obvious consequences of high saline conditions are increased accumulation of ROS, ionic imbalance and osmotic stress. Many studies have comprehensively explored the protein phosphorylation levels under salt stress. It has been reported that upon 24 hr treatment of rice with 50mM NaCl, the phosphorylation level of four important proteins namely cytoplasmic malate dehydrogenase, calreticulin and others were found to increase by two-dimensional gel electrophoresis (2DE) in-gel kinase assay [119]. The cytoplasmic malate dehydrogenase plays an important role in glycogen metabolism and energy production whereas calreticulin is a key $Ca^{2+}$ binding protein and signaling molecule. Similarly, it has been shown that

**Table 2. Listing of the bio-informatics tools available to identify the phosphorylation sites on proteins.**

| Name | Description | References |
|---|---|---|
| SAPHIRE<br>http://yeoman.usask.ca. | Applies phosphorylation data from other organisms to enhance the accuracy of predictions in a target organism | [95] |
| ConDens | Uses an evolutionary model as well as conservative motif matches rather than individual alignment of phosphorylation sites | [99] |
| PhosphoSitePlus<br>(http://www.phosphosite.org) | Gives detail about structure and regulatory interactions of phosphorylation sites. It has reported about 500 human and mouse proteins | [100] |
| NetPhosBac 1.0 | Bacterial serine and threonine phosphorylated sites prediction tool | [101] |
| PhosphoPep v2.0<br>(http://www.phosphopep.org/) | Source for phosphorylation database for Model organisms | [102] |
| GPS 2.0 | Prediction of kinase specific phosphorylation sites in a same group/Subfamily for recognizing similar sequence patterns of substrates for modification | [103] |
| PhosphoPOINT<br>(http://kinase.bioinformatics.tw/) | A human kinome interactive database | [104] |
| PhosPhAt 3.0 | Plant specific (*Arabidopsis*) phosphorylation prediction tool | [92] |
| NetPhosYeast 1.0 | Yeast serine, threonine phosphorylation sites are predicted | [105] |
| PHOSIDA<br>http://www.phosida.com/<br><br>Modification of Database<br>'PHOSIDA 2011 | Allows retrieval of different PTMs such as phosphorylation, acetylation for different protein of interest. It also provides evolutionary as well as structural information. | [106]<br>[107] |
| NetworKIN 1.0<br>http://networkin.info/search.php | Helps in retrieval of *in vivo* kinase-substrate network | [108] |
| Phospho.ELM 8.3 (PhosphoBase)<br>(http://phospho.elm.eu.org) | Provides information about exact position of phosphorylation of the substrate for kinases | [109]<br>[110] |
| PlantsP<br>(http://PlantsP.sdsc.edu.) | Information on a collection of T-DNA insertion mutants (knockouts) in each protein kinase and phosphatase in *Arabidopsis thaliana* | [111] |
| ScanProsite<br>http://www.expasy.org/tools/scanprosite/ | Provides web interface about results from PROSITE | [112] |
| NetPhos 2.0<br>(http://www.cbs.dtu.dk/services/NetPhos/) | Neural network-based method for predicting potential phosphorylation sites at serine, threonine or tyrosine residues in protein sequences | [113] |
| PhosphoNET<br>(http://www.phosphonet.ca/) | Provides data about 23000 human phosphorylation sites | University of British Columbia<br>And Simon Fraser University |

under high salt (200mM) conditions, protein H of photosystem II reaction center is phosphorylated [120]. Photosystem II phosphorylation contributes to the functional folding of complex and bulky photosynthetic membranes in *Arabidopsis* and imparts sustained photosynthetic activity [121]. Therefore, the significant increase in the phosphorylation status of protein H of photosystem II reaction center may perform similar function (in providing sustained photosynthetic activity) in salt-treated rice.

In maize, roots exposed to 25mM salt treatment for 1h induced phosphorylation status of around ten proteins including fructokinase and UDP-glucosyltransferase BX9 [122]. Additional phosphoproteomics results were obtained by modulating concentration and duration of the stress treatment using ESI-Q-TOFMS with $Ti^{4+}$-IMAC enrichment technique [123]. The majority of the phosphorylated proteins were implicated in metabolic processes, cellular functionality, transport, photosynthesis and responses to stress. In addition to this, these proteins are involved in $Ca^{2+}$/calmodulin signaling pathway in mediating salt stress responses and tolerance in plants.

The well characterized SOS pathway designated to be the key pathway for maintenance of cellular ionic homeostasis, was the first CBL-CIPK pathway identified in plant cells

[124-130]. The SOS1 ($Na^+/H^+$ antiporter) localized at the plasma membrane [131] is vital to enhance salt tolerance. External environmental stimulus in the form of salt stress activates the AtCBL4/SOS3-AtCIPK24/SOS2 complex to stimulate the activity of SOS1 [132]. Consequently, excess $Na^+$ present in the cytosol is excluded into the soil [133]. Reports also suggest that tonoplast localized $Na^+/H^+$ (NHX) antiporters are activated by AtCIPK24/SOS2 with the involvement of AtCBL10. The activated NHX sequester excess $Na^+$ into the vacuole [134].

Similar to CIPKs, $Ca^{2+}$-dependent protein kinases (CDPKs) are also involved in many biotic and abiotic stress responses. So far, most of the CDPK substrates described by using *in-vitro* assays are involved in an array of cellular processes such as primary metabolism and secondary metabolism, stress responses, ion transport, water transport, transcription and signaling [135-138]. Yeast two-hybrid assays with different CDPK variants have enabled identification of putative substrates [139-141]. However, these putative interactions need to be confirmed *in-vivo*. Many transcription factors (TFs), including ABF4 (ABA-responsive element-binding factor 4), RSG (repression of shoot growth) and HsfB2a (heat shock factor B2a) have been characterized in detail as CDPK substrates. ABF4 is involved in ABA signaling [142], RSG in gibberellin signaling [143, 144] and HsfB2a in herbivore-induced signaling [145].

The 34 *CDPK* genes that have been identified in *Arabidopsis thaliana* [146, 147] and 29 *CDPK* genes have been found in *Oryza sativa* (rice) [148]; many of them have been implicated in biotic and abiotic stresses [149]. AtCPK3 and AtCPK6 have been reported to be involved in the abiotic stress and ABA signaling pathways. CPK3 and CPK6 regulate guard cell ion channels [150]. Furthermore, overexpression of CPK6 led to enhanced tolerance to salt and drought stresses implying that CPK6 is a positive regulator of these stresses [151]. Similarly, AtCPK10 is involved in tolerance to drought stress [152].

*Arabidopsis* CDPKs mediate ABA signaling pathway by phosphorylating ABA-responsive element binding factors (ABFs), which are in fact a subfamily of basic leucine zipper class transcription factor (TF) proteins. The AtCPK4 and AtCPK11 are involved in the ABA-regulated physiological processes such as seed germination, seedling growth, stomatal movement and tolerance to salt and drought stresses. CPK4 and CPK11 phosphorylate two ABA-responsive TFs, ABF1 and ABF4, suggesting that they positively regulate the CDPK-mediated ABA signaling pathway [153]. Moreover, AtCPK32 interacts with ABF4 and phosphorylates ABF4 in kinase assay and the overexpression lines of CPK32 exhibited salt sensitivity implying that CPK32 contributes in ABA and stress responses by regulating ABA-responsive gene expression through ABF4 [154]. Furthermore, overexpression of one of the rice CDPKs, OsCDPK7 has been shown to enhance tolerance to cold, salt and drought [155]. OsCDPK13 overexpression increases cold tolerance [156] while OsCPK21 overexpression causes increased tolerance to salt stress and sensitivity to ABA [157].

Potato CDPK4 and CDPK5 (StCDPK4 and StCDPK5) have been reported to modulate reactive oxygen species (ROS) production by phosphorylating NADPH oxidase [158]. Likewise, AtCPK5/AtCPK6 and CPK4/CPK11 seem to regulate ROS production [159]. OsCPK12 has been reported to be positively regulating tolerance to salt stress by reducing the accumulation of ROS level. OsCPK12 does so by controlling the expression of *OsAPx2, OsAPx8* and *OsrbohI* [160].

Though many MAP kinases are regulated positively under abiotic stresses, some of them also play roles as negative regulators. For instance, OsMAPK33 overexpression in rice results in high sensitivity to salt stress [161] but AtMKK9 overexpression causes salt sensitivity in *Arabidopsis* [162]. The mechanism of salt tolerance or sensitivity by MAPK cascade is not much explored and their targets are not identified. In a high-throughput in-solution kinase assay, 48 potential targets of MPK3 and 39 of MPK6 have been identified [163]. However, detailed *in-vivo* characterization by different methodologies is required to validate the interactions and understand the physiological relevance of these interactions.

### 6.2. Drought

Scarcity of water for a long time leads to drought, which is more frequent and prevalent under unprecedented climate change. Physiologically, plants manifest drought condition with leaf rolling, reduced photosynthetic rate, slower growth, and pollen sterility leading to yield losses. Exposure to drought induces a wide range of molecular and cellular responses. This culminates into osmotic adjustments, ABA accumulation and altered protein turn over [164, 165]. Phosphoproteomic analysis of drought stressed maize seedlings by iTRAQ-based quantitative approach [166, 167] enabled identification of more than 100 leaf proteins showing altered phosphorylation level in maize seedling when subjected to PEG treatment. These proteins mostly belong to the class of HSPs, kinases, phosphatases and transporters; primarily involved in photosynthesis, response to stimulus and signal transduction [165]. In Chickpea (*Cicer arietinum*) seedlings, a total of 91 dehydration-responsive phosphoproteins have been reported out of which 34 proteins showed enhanced phosphorylation when subjected to drought stress [168]. Most of these proteins were involved in mediating plethora of cellular functions ranging from cell defense and immunity, photosynthesis, photorespiration, molecular chaperones to ion transport.

As a key stress hormone ABA mediates plant responses to drought and salinity. AtCPK10 and AtCPK30 were the first CDPKs identified as positive regulators of the barley stress- and ABA-inducible *HVA1* promoter in maize protoplasts [169]. Moreover, AtCPK32 is reported to activate ABF4 *in-vivo*, causing the induction of ABF4 target genes [170]. In addition to this, ABF1 and ABF4 regulation by AtCPK4 and AtCPK11 under ABA, suggest the role of these kinases in long-term adaptation [171].

AtCIPK26 interacts with AtCBL1/9 to regulate the *Arabidopsis* NADPH oxidase in the ROS pathway [172]. PPI methods like yeast two-hybrid, *in-vitro* pull down and BiFC confirmed that AtCIPK26 interacts with the RING-type E3 ligase and Keep on Going (KEG) leading to ubiquitin-mediated degradation of the latter [173]. KEG is known to target the group A bZIP protein ABA-insensitive 5 (ABI5) for degradation and thus negatively regulating the ABA sig-

naling pathway [174, 175]. Interaction of AtCIPK26 with ABI1, ABI2 and ABI5 positively regulates the ABA signaling [173].

Many transcriptional and biochemical analyses done so far have given a hint toward the involvement of MAP kinases in water scarcity stress. For example, AtMPK6 is essential to increase plant tolerance to dehydration by regulating the RNA decapping activity [176]. The transcriptional regulatory dynamic analyses of *Arabidopsis* MAP kinases have revealed that MPK2, MPK3, MPK4, MPK5, MPK12 and MAPKKK4 are up regulated under dehydration [177]. One of the rice MAP kinases, OsMAPK5 overexpression contributed to enhanced drought tolerance [178] implying the positive regulation of drought stress tolerance by OsMAPK5.

### 6.3. Flooding

With the continuous global climatic change, the frequency of heavy rainfall and flooding is expected to increase [179]. Flooding has grave negative effects on normal growth and development of nearly all crops [180] as it causes oxygen concentration in the soil to go down [181] and thus interferes with mitochondrial oxidative phosphorylation. Proteome investigation of flood-stressed crop has revealed that phosphorylation and N-glycosylation are the most common PTMs under this stress.

Soybean is one of the important crops and it is sensitive to flood stress. The phosphoproteome analysis of 3h flood treated soybean roots showed that several ribosomal proteins and the carbohydrate metabolism related proteins are majorly involved in inducing phosphorylation levels [182].

*OsCIPK15* under $O_2$-deficiency regulates *Ramy3D* in rice [183]. *Ramy3D* is one of the members of a gene family encoding α-amylases and it plays crucial role in anaerobic germination [184]. Moreover, the submergence 1A (*Sub1A*) is important in flood-tolerant phenotype. Reports showed that OsCIPK15 in involved in the up-regulation of *Ramy3D* causing elongation in *Sub1A* mutant and allowing starch degradation under $O_2$-deficient condition [185].

Recently, a study provided the evidence that the activation of OsMPK3 in submergence is SUB1A dependent [186]. The study further establishes that SUB1A1 works with MPK3 in a positive regulatory loop to regulate submergence tolerance. OsMPK3 phosphorylates SUB1A1; SUB1A1 specifically interacts with the promoter of MPK3 and modulates its expression. Methods like yeast two-hybrid, BiFC, *in-vitro* kinase assay validated the interaction between MPK3 and SUB1A1. The *in-vitro* kinase assay indicated that MPK3 is specifically involved in the phosphorylation-coupled activation of SUB1A1 and this in turn leads to submergence-tolerance trait [186].

### 6.4. Heat

Continued heat wave can cause shedding of leaves, inhibits flower and fruit formation and even death of the complete plant [187, 188]. Plants, when subjected to heat stress activate multiple pathways and adaptive circuitry in response [189]. PTMs contribute immensely in these responses and protein phosphorylation has been investigated a number of times with respect to stress conditions. Rice plants have been shown to reprogram the phosphoproteome under heat stress [190]. Under heat stress, there is a marked change in the phosphorylation status of many proteins in rice leaves. The dephosphorylation of RuBisCO and the ATP synthase phosphorylation caused reduction in the activities of RuBisCO and ATP synthase, respectively.

### 6.5. Cold

More than 30% of the global arable land area is encountering cold stress [191]. Many important crops *e.g.* rice, maize, soybean, cotton are more prone to cold stress and are incapable of acclimation. Cold stress can have severe impacts on crop plants as it inhibits soil water absorption, induce cellular dehydration and lead to yield loss [192]. In a report, glyoxalate-I, calreticulin and calmodulin-related proteins from leaf sheath showed an enhanced phosphorylation at 5ºC for 24 h [193]. In the roots of rice plants, differential phosphorylation of a total of 12 different proteins has been shown [194]. Among these, the major population of the proteins was involved in the carbohydrate metabolism and signal transduction.

AtCIPK7 forms a complex with AtCBL1 and plays a role in the cold response [195]. Among rice CIPKs, OsCIPK03 function as a positive regulator of cold tolerance [196]. CBFs (cold responsive element-binding protein factors) are key transcriptional regulators required for cold tolerance. Although, the role of CDPKs in cold stress response mostly remains elusive, OsCPK13 (OsCPK7) is reported to impart cold tolerance without affecting transcriptional regulation [197]. Similarly, AtCPK1 regulates the level of phosphoproteome after cold treatment [198] and overexpression of OsCPK7 (OsCDPK13) stimulates cold tolerance and the accumulation of the cold-responsive chaperone calreticulin [199, 200]. These reports clearly indicate that CDPKs are involved in cold tolerance; however the molecular mechanisms remain to be discovered.

### 6.6. Nutritional Deficiency

Both micro- and macronutrients are essential for proper growth and development of the plants. Potassium ($K^+$) is the most abundant cation in plants and plays essential roles in a number of physiological and biochemical reactions [201]. Plants have evolved mechanisms to take up sufficient $K^+$ to meet their demand. However, under low $K^+$ condition one of the Calcineurin B-like protein Interacting Protein Kinase (CIPK23) activates a regulatory pathway for $K^+$ uptake in *Arabidopsis* [202, 203]. AtCIPK23 is a Ser/Thr protein kinase that phosphorylates AKT1 and enhances $K^+$ uptake, particularly, under low $K^+$ stress [202, 203]. The AtCIPK23-AKT1 interaction has been validated and confirmed with yeast two-hybrid assay, BiFC and *in-vitro* phosphorylation. Moreover, AtAKT1 plays essential role not only in $K^+$ uptake but also in stomatal movement and response to water stress [204].

In addition to the specific interaction between AtAKT1 and AtCIPK23; AtCIPK9, which is the closest homolog of AtCIPK23, can also interact with AtCBL3 to regulate $K^+$ uptake and homeostasis under low $K^+$ stress [205, 206]. Moreover, AtCIPK6 and AtCIPK16 both have been reported

to activate AtAKT1 by interacting with specific AtCBLs [206]. Besides AtAKT1 activation under low $K^+$ stress, AtCIPK23 may also regulate CHL1 by phosphorylating T101. CHL1 (AtNRT1.1) functions as a dual-affinity nitrate transporter [207]. One of the speculated regulatory mechanisms of CHL1 is by phosphorylation [208]. The phosphorylated CHL1 functions as a high affinity transporter [208]. On the basis of a differential transcriptomic study [209], it has been shown that AtCIPK8 is involved in regulating the primary nitrate response in the *chl1-5* mutant; however the exact mechanism of CHL1 regulation by AtCIPK8 is still not clear [209].

Briefly, the activation of the plant kinase cascades and its outcome has been summarized in the form of model (Fig. **2**).

### 6.7. Biotic Stress

Plants being sessile and anchored in the soil constantly encounter harmful pathogens throughout their life. For successful survival, plants must sense the potential threat in the form of invading pathogens and mount the defense mechanism [210-212]. Pathogen/microbe-associated molecular patterns (PAMPs/MAMPs) or pathogen released effector proteins evoke many early defense responses such as $Ca^{2+}$ flux, mitogen-activated protein kinases (MAPKs), production and accumulation of ROS and induction of ethylene biosynthesis. The cumulative effect of these, help plants to ward off the invading pathogens.

Plant Pattern Recognition Receptors (PRRs) are able to recognize both pathogen-released external PAMPs/MAMPs and Damage-Associated Molecular Pattern (DAMPs) released after pathogen invasion and this initiates PAMP-Triggered Immunity (PTI) [213]. Plants use resistance (R) proteins to detect the presence of pathogen-derived effectors and thus trigger Effector-Triggered Immunity (ETI) [213]. The MAPK activation cascade is one of the early events in the PTI and ETI [213]. Activation of MAPK cascade leads to the phosphorylation of many target proteins like transcription factors and enzymes involved in the synthesis of antimicrobial metabolites, signaling of defense hormones and hypersensitive response (HR) (reviewed in detail by [213]). Briefly, the activation of the MAPK cascade and its outcome has been summarized in the form of a model (Fig. **3**).

## CONCLUSION AND FUTURE PERSPECTIVE

The progress in the comprehensive understanding of many crucial signaling cascades initiated by the activation of kinases in plants is hampered by the unavailability of in-depth information about the actual physiological substrates. Mounting evidence suggested that protein kinases are involved in instant stress signaling reactions as well as in the long-term adaptive responses. While some rapid stress signaling targets of these kinases have been identified but the nature of substrates mediating adaptive responses is still at infancy stage and needs to be investigated.

Though we have come a long way in terms of information about the targets of many of the kinases in plants, still a lot has to be done and achieved. It is noteworthy that most of the information that we have generated is from model plant, *Arabidopsis*. The need of the hour is to decipher the targets of kinases from rice, wheat, barley and other plants of eco-

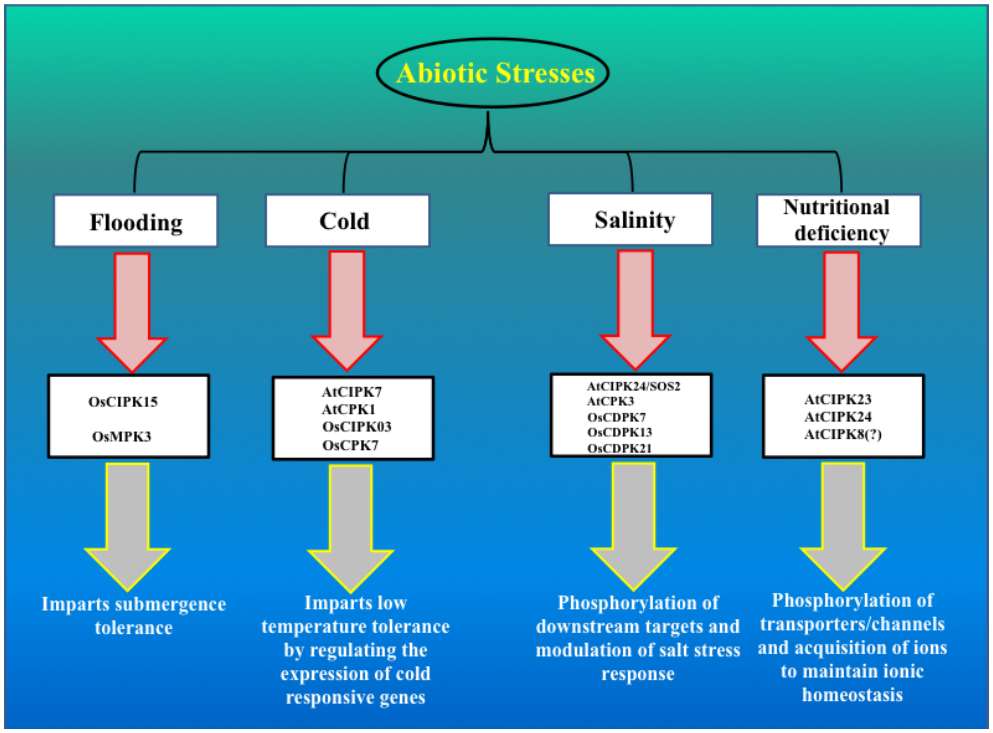


**Fig. (2).** A general outline of abiotic stress responses and implication of kinases therein. Responses against abiotic stresses such as flooding, cold, salinity, nutritional deficiency and others bring about many plant kinases in signaling map. These kinases impart adaptation and tolerance against these stresses through phosphorylating the downstream target proteins which includes transcription factors (TFs), enzymes *etc*.

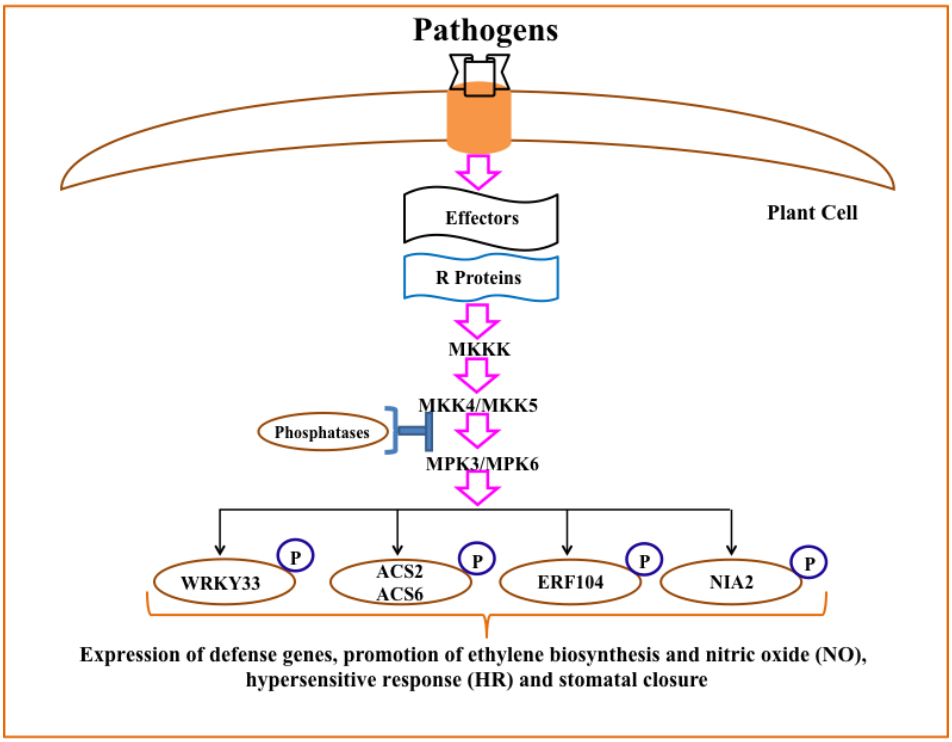


**Fig. (3).** A general outline of biotic stress responses and implications of MAP kinases therein. Activation of plant Mitogen-Activated Protein Kinase (MAPK) cascade is one of the earliest signaling events in PTI (Pathogen-Associated Molecular Patterns (PAMPs) triggered immunity) and ETI (Effector Triggered Immunity). Phosphorylation-the most common post-translational modification is mediated by these MAPKs and phosphatases, which fine-tune these signaling events. The phosphorylation of target proteins, which include transcription factors, enzymes *etc*. promotes synthesis/or signaling of defense hormones, activation of defense genes, synthesis of nitric oxide (NO) to mediate stomatal closure, hypersensitive responses (HR)-like cell death and other defense responses.

nomic importance. Thorough knowledge will enable to develop potential and promising biotechnological tools to generate high yielding and stress tolerant crops for future.

## LIST OF ABBREVIATIONS

| | | |
|---|---|---|
| CBL | = | Calcineurin B-like protein |
| CIPK | = | CBL-interacting protein kinase |
| DAMP | = | Damage-associated molecular pattern |
| ETI | = | Effector-triggered immunity |
| HR | = | Hypersensitive response |
| MAPKs | = | Mitogen-activated protein kinases |
| NO | = | Nitric oxide |
| PAMP | = | Pathogen-associated molecular pattern |
| PTI | = | PAMP-triggered immunity |
| TAP | = | Tandem affinity purification |

## CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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