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Mitogen Activated Protein Kinases: A Hunt for their Physiological Substrates in Plants

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ABSTRACT

Mitogen activated protein kinases (MAPKs) are important signal transducing enzymes that connect various sensors/receptors to a wide range of cellular responses in mammals, yeast and plants. The MAPKs are part of a phospho-relay cascade, which essentially consists of three components namely MAPK kinase kinase (MAPKKK), MAPK kinase (MAPKK) and MAPK. They are connected to each other by an event of phosphorylation. MAPK, the last component of the cascade, upon activation phosphorylates variety of cytosolic and nucleic proteins for appropriate cellular reorganization. In plants MAPK consist of a multigene family having twenty and sixteen members in Arabidopsis and rice, respectively. Though search for the substrate of MAPK in plants is on, there are only a few reports of phosphorylation of downstream targets by activated MAPK. In the present review we take an overview of the progress made in identifying the substrate of MAPK in plants, the approaches undertaken and finally discuss the future perspectives in hunt for the putative substrates.

Keywords: *in-vitro* phosphorylation assay, MAP kinase, protein microarray, protoplast assay, yeast two-hybrid analysis

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INTRODUCTION

The signaling cascades operational in the plants play a vital role in conferring resistance to the sessile plants besides carrying out the normal growth and developmental cues. The most important signaling pathway in this regard is Mitogen Activated Protein Kinase (MAPK) cascade. MAPK pathway is one of the primary signaling cascade coordinating the survival cues inside the plant. A canonical MAP kinase pathway minimally consists of a three tier phosphorelay module namely MAPKKK-MAPKK-MAPK which connect a diverse developmental and defence signals to the appropriate transcriptional response (Sinha *et al.* 2011). After perception of the elicitor, receptor mediated activation of a MAPKKK can occur through physical interaction and/or phosphorylation by the receptor itself, intermediate bridging factors or interlinking MAPKKKKs. MAPKKKs activate downstream MAPKKs through phosphorylation on two serine/threonine residues in a conserved S/T–X3-5–S/T motif. On the other hand MAPKKs are dual-specificity kinases that phosphorylate MAPKs on threonine and tyrosine residues in the T–X–Y motif. MAPK, the last component upon activation phosphorylates a variety of cytosolic and nuclear substrates including transcription factors, protein kinases and cytoskeleton- associated proteins. These MAPK substrates are direct regulators of transcriptional programming for appropriate cellular reorganization. The specificity of different MAPK cascades functioning within the same cell is generated through the presence of docking domains found in various components of MAPK modules and possible scaffold proteins (Rodriguez *et al.* 2010).

Our knowledge on plant phosphoproteins in general and mitogen activated protein kinases in particular has increased tremendously over the period of time but unfortunately the substantial knowledge regarding their downstream substrates is still lacking. This has put a brake in advancing our knowledge of cell signalling. The scenario in case of plants is still worse than animal systems. The primary cause for this information drag is because of the unavailability of reliable and authentic techniques. The other reasons may include the broad experimental manipulation of these cel-

Received: 1 January, 2012. Accepted: 25 December, 2012.

Invited Mini-Review

Table 1 Approaches employed for plant MAPK substrate identification.

Technique	Methodology	Pros and Cons
1. In-tube kinase assays	An *in-vitro* phosphorylation assay where purified MAPK and the possible substrate protein are incubated in presence of kinase buffer containing [γ-^{32}P]ATP. The possible substrates can be shortlisted on the basis of yeast two-hybrid (Y2H) or bi-molecular fluorescence complementation (BiFC) interaction screening methods.	**Pros:** Useful and easy method **Cons:** Random fishing approach, low throughput, high background noise.
2. Protein microarrays	Chip based method where the possible MAPK substrate proteins translated either *in-vitro* or *in-planta* are blotted on the chips. The chips are then incubated with purified MAPKs in the presence of kinase buffer containing $MgCl_2$ and radio-labelled ATP. Potential substrates are detected by autoradiography of the microarrays.	**Pros:** High throughput, sensitive, systematic approach, phosphorylation site identification. **Cons:** Promiscuous activity of MAPK substrates on chip, masking of phosphorylation sites on chip, expensive, stringent controls needed.
3. *In-vivo* assays	Antibody based method where proteins phosphorylated in the consensus phosphorylation site by the kinase can be detected by western blot analysis. Here two types of antibodies namely phosphospecific and phospho-motif specific antibodies are used.	**Pros:** *In-vivo* technique, no problems of proper phosphorylation site priming, no problems with proper post-translational modification of proteins. **Cons:** Hard to develop particular phosphospecific antibodies as MAPK substrate interaction domain in plants are not fully defined.

lular networks which often prove lethal or associated with multiple phenotypes. Also the laborious and expensive nature of the already available techniques may also hamper the scientific efforts in this direction. Here we will first give an account of the traditional techniques used for MAP kinase substrate identification and then discuss the modern high throughput technologies which are being used or can be used in plant systems. Lastly, the progress made in deciphering the MAPK substrates will be discussed.

MAPK substrate identification techniques

Several efforts carried out to find out the MAPK substrates, can be broadly categorized into the following categories (see **Table 1** for comparison):

A. ***In-vitro*** **phosphorylation approaches:** This broadly includes two sub-categories:

1. Kinase assays using in-tube reactions.
2. Kinase assays using protein filters or protein microarrays.

B. ***In-vivo*** **phosphorylation approaches:** This mainly includes usage of phospho-specific antibodies.

A. Identification of MAPK substrates by *in-vitro* approaches

1. Kinase assays using in-tube reactions

The commonly used *in-vitro* technique is to incubate a purified MAPK with a putative purified substrate protein in a kinase buffer usually containing $MgCl_2$ and [γ-^{32}P]ATP. The substrate here is purely guessed based on the background literature but it cannot be used where the information regarding the substrate is totally lacking. Random fishing approaches can also be used where total plant crude extract can be used against a candidate MAPK for *in-vitro* kinase assay and later the individual signals can be identified using mass spectrometry. The problem of background noise can be reduced by using $MnCl_2$ in place of $MgCl_2$ provided the Mn^{2+}-ATP is compatible for the substrate kinase reaction (Kersten *et al.* 2009).

The other way to identify the MAPK substrates is to find their interacting partners and then validate the individual interactions by *in-vitro* kinase assays. This can be done by carrying out a global yeast two-hybrid (Y2H here onwards) study followed by *in-vitro* kinase assay of the purified protein interacting partners. (Lee *et al.* 2008). *Arabidopsis* AtMPK4 substrate MKS1 (Andreasson *et al.* 2005) or the tobacco transcription factor WRKY1, were identified by a targeted Y2H approach as a potential substrate of SIPK, a MAPK of tobacco (Menke *et al.* 2005). The major drawback of this approach is the encounter with lots of false positives and on the other side many protein-kinase interactions are not detected by Y2H assays. Also it should be kept in mind that during global Y2H screening a kinase does not interact with its substrates only (Ptacek *et al.* 2005). For this reason, using bimolecular-fluorescence complementation (BiFC) assay in place of Y2H is getting popularity these days in deciphering the kinase-substrate interactions (Pusch *et al.* 2011).

The other more specific *in-vitro* strategy is 'chemical genetics' approach using Shokat mutants. In this technique, a functionally silent mutation is engineered into the ATP binding site of the kinase of interest by replacing a conserved bulky hydrophobic amino acid with a small amino acid (e.g. glycine or alanine) and thus creates an enlarged but functional ATP-binding pocket that uniquely enables the mutant to use an ATP analogue that has a bulky side chain attached (e.g. N6-[phenethyl] ATPγS). In presence of the ATP analogue only the mutant kinase will be active. This method has proven very effective in reducing the background phosphorylations. This technique has been recently used to find novel substrates of human AMPKα2 (Banko *et al.* 2011) and plant CDPKs (Boehmer and Romeis 2007).

2. Kinase assays using protein filters or protein microarrays

Although all the above mentioned techniques are very useful but the systematic approach to hunt MAPK substrates needs high throughput techniques. The most significant methods used in this regard are chip based methods where proteins are blotted on the chips translated either *in-vitro* or *in-planta* (Popescu *et al.* 2009). Protein filters and protein microarrays are increasingly applied to test the phosphorylation of thousands of proteins by a specific kinase on a solid phase in parallel. The additional advantages include subsequent analysis of phosphorylation sites in the identified substrates and the deduction of consensus sites for specific kinases. Either protein filters containing immobilized expression libraries or the protein microarrays with purified recombinant proteins can be used. The filters/microarrays are then incubated with purified MAPKs in the presence of kinase buffer containing $MgCl_2$ and radio-labelled ATP. Potential substrates are detected by autoradiography of the filters/microarrays.

Though there are some excellent reports of protein microarray from non-plant systems but here we will discuss only plant based approaches. The first report came from the study on barley (Kramer *et al.* 2004) and later on *Arabidopsis* (Feilner *et al.* 2005). In both the studies, bacterially expressed HIS-tagged proteins were non-covalently immobilized on NC-derived polymer based FAST™ slides. In the barley (*Hordeum vulgare* cv. 'Barke') study potential substrates were selected only based on qualitative evaluation of the radioactive signals but in *Arabidopsis* study a well defined threshold was set after quantitative evaluation

of the phosphorylation signal. Microarray-based testing of 1690 *Arabidopsis* proteins by Feilner *et al*. (2005) identified 48 *in-vitro* substrates for AtMPK3 and 39 for AtMPK6 containing a largely overlapping set of 26 candidates.

The usefulness of protein arrays in simple and sensitive high throughput interaction screening studies of plant proteins was demonstrated by Popescu *et al.* (2009). They used ten different *Arabidopsis* MAPKs, activated by MAPKKs *in-vivo*, to probe high-density protein microarrays to determine their phosphorylation targets. The analyses revealed known and novel signaling modules encompassing 570 MAPK phosphorylation substrates out of 2158 proteins. These substrates were enriched in transcription factors involved in the regulation of development, defence, and stress responses. Transient *in-planta* activation of some of these transcription factors (TGA1, WRKY6, WRKY8, WRKY53, WRKY62, WRKY65) was also demonstrated. Interestingly, AtMPK6 was found to have more *in-vitro* substrates than any of the other MAPKs, perhaps reflecting the frequency with which this MAPK is activated by upstream MAPKKs. The extensive overlap between AtMPK3 and AtMPK6 substrates found by Feilner *et al*. (2005) and Popescu *et al.* (2009) can be contributed to the diversity of activators, as well as the paralogous relationship between the two MAPKs. The other aspect of these studies is their inability to detect the already documented kinase-substrate candidates. Although WRKY22 and WRKY29 transcription factors were earlier implicated as operating downstream of AtMPK3 and AtMPK6, direct interactions between these WRKYs and any MAPKs have yet to be shown (Asai *et al.* 2002). The possible explanations for the false negative rate (FNR) in these studies are: (i) the amount of protein spotted on the array was too low to be phosphorylated by the kinase, (ii) masking of a phosphorylation site by protein immobilization, (iii) abolished interaction of the protein with the kinase, and (iv) altered kinetics of the phosphorylation reaction on the array surface. Furthermore, the setting of the threshold for a positive signal evaluation on different microarray surfaces influences the FNR considerably. Nevertheless, the FNR must be considered in light of the advantage of the array format and the ability to rapidly survey thousands of proteins in a single experiment with low material requirements (Kersten *et al*. 2009).

Vlad *et al.* (2008) implemented a semi-degenerate peptide array screen to define the phosphorylation preferences of four CDPK and Snf1-related kinases from *Arabidopsis thaliana*. Here biotin-tagged peptides are arrayed in multiwell plates and incubated in solution with the kinase of interest and radio-labelled ATP. Reactions are then spotted simultaneously onto a streptavidin membrane, which is washed, dried, and analyzed by autoradiography or phosphor imaging. This approach is a faster, more sensitive, and more generally applicable method for determining kinase phosphorylation motifs than older peptide library screening approaches based on Edman sequencing. All *in-vitro* phosphorylation assays were performed using large amount of radio-labelled ATP. In place of using hot ATP a few studies have been performed using Pro-Q DPS for the detection of phosphorylation events on microarrays enabling the use of a common microarray scanner for the quantitative evaluation of the microarrays (Martin *et al.* 2003).

Although the *in-vitro* approaches are highly informative, they carry some serious drawbacks. First, protein kinases are well known to show promiscuous activity *in-vitro*. Second, proteins expressed in prokaryotic systems might not fold appropriately or carry the necessary post-translational modifications (e.g. phosphorylation sites might not be primed). Third, these approaches require the phosphorylated substrate under conditions that do not necessarily faithfully represent those experienced by the substrate and the kinase in a cell; that is, cofactors or binding proteins which are important for the *in-vivo* phosphorylation may be missing under *in-vitro* conditions. Last, the subcellular compartmentalization of the kinase and substrate proteins *in-vivo* might hamper a protein substrate from becoming effectively exposed to the kinase, even though that protein might be an excellent substrate *in-vitro* (Berwick and Tavare 2004).

B. Identification of MAPK substrates by *in-vivo* approaches

The *in-vitro* data should be validated in intact cells under physiologically relevant conditions. The *in-vivo* experiments should be set which will analyse the phosphorylation status of the protein *in-planta* and also confirm the link between the activation of the kinase and the phosphorylation of the substrate protein. But this job is far more complicated to do than said because a cell is a mixed bag of lots of kinases having overlapping substrates.

As we know that MAPKs are serine/threonine kinases which distinguish a sequence specific phospho-motif in their substrates. For the detection of specific phosphorylation events anti-phospho antibodies that recognize such phospho-motifs can be applied. The sequences of *Arabidopsis* MAPK substrates contain the phosphorylated serine followed by proline which is in agreement with previous reports that [S/TP] is the minimal consensus motif for MAPK phosphorylation (Sharrocks *et al.* 2000).

Two types of antibodies namely phosphospecific and phospho-motif specific antibodies are being used for *in-vivo* MAPK substrate phosphorylation studies. Phosphospecific antibodies recognize a specific phosphorylated sequence of an individual protein, while phospho-motif antibodies recognize a single amino acid residue in the context of a protein, but only when the residue is phosphorylated. Proteins phosphorylated in the consensus phosphorylation site by the kinase can be detected by western blot analysis of cell lysates, subcellular fractions, or fractions that have been purified by affinity chromatography or immunoprecipitation. Phospho-motif antibodies particularly phosphoserine- and/or phosphothreonine-specific antibodies tend to be rather poor at immunoprecipitating their binding partners, while phosphotyrosine antibodies in finding tyrosine kinase substrates have proved substantially successful in animal systems. A possible reason for the failure is that the phosphoserine and phosphothreonine are components of some secreted proteins and are thus less immunogenic than phosphotyrosine, which would make antibody generation towards phosphotyrosine more feasible. Also there are no clear evidences of MAPK substrate interaction domain in plants suggesting a possible role of more remote regions of the protein in determining whether a given PxSP site will be phosphorylated by a MAPK or not. Andreasson *et al.* (2005) found MKS1 protein as the phosphorylation substrate of *Arabidopsis* AtMPK4 and showed the levels of phosphorylated MKS1 detected with a phosphoserine/phosphothreonine-specific antibody were markedly higher in wild-type plants than in *mpk4* mutants.

By combining the *in-vitro* and *in-vivo* strategies some basic criteria can be set for formal identification of a novel protein kinase substrate:

1. The protein kinase should phosphorylate the purified substrate *in-vitro* either with or without priming phosphorylation events.

2. The *in-vitro* stoichiometry of phosphorylation should be significant, preferably approaching 1 mol of phosphate for every 1 mol of phosphorylation sites on the substrate protein. However the stoichiometry in intact cells might be low even though the phosphorylation event is physiologically relevant.

3. Assuming the same subcellular compartmentalization of kinase and substrate, the protein substrate must be phosphorylated in intact cells in response to stimuli that activate the protein kinase with a similar stimulus dose–response and time course.

4. The phosphorylation of the protein substrate must be on the same site *in-vitro* and *in-planta.*

5. Constitutively active mutants of the protein kinase must stimulate phosphorylation of the protein substrate.

Table 2 List of MAPKs and their substrates.

MAPK	Substrate	Plan system	Reference
AtMPK4	MKS1	Arabidopsis	Andreasson *et al*. 2005
AtMPK3, AtMPK6	WRKY33	Arabidopsis	Mao *et al*. 2011
AtMEKK1	WRKY53	Arabidopsis	Miao *et al*. 2007
10 AtMPKs	570 substrates	Arabidopsis	Popescu *et al*. 2009*
AtMPK3	48 substrates	Arabidopsis	Feilner *et al*. 2005
AtMPK6	39 substrates	Arabidopsis	Feilner *et al*.2005
AtMPK3, AtMPK6	WRKY22, WRKY29	Arabidopsis	Asai *et al*. 2002
SIPK	WRKY1	Tobacco	Menke *et al*. 2005
SIPK, NTF4, WIPK	WRKY8	Tobacco	Ishihama *et al*. 2011
AtMPK3	VIP1	Arabidopsis	Djamei *et al*. 2007
AtMPK3, AtMPK6	SPCH	Arabidopsis	Lampard *et al*. 2008
AtMPK3, AtMPK6	ERF5	Arabidopsis	Son *et al*. 2012
AtMPK3, AtMPK4	EIN3	Arabidopsis	Yoo *et al*. 2008
AtMPK6	ERF104	Arabidopsis	Bethke *et al*. 2009
AtMPK6	ACS6	Arabidopsis	Liu and Zhang 2004

*Popescu *et al*. reported WRKY53 as one of the common substrates for AtMPK6, AtMPK7, AtMPK10.

6. Dominant-negative and kinase inactive forms of the protein kinase (assuming that the kinase in question is the only kinase that can phosphorylate the substrate at the site under investigation *in-vivo*) must inhibit phosphorylation of the protein in response to the stimulus.

Progress in elucidation of plant MAPK substrates

It is difficult to identify the MAPK substrates because of the structural and evolutionary diversity of MAPK substrates and also the transient nature of the MAPK-substrate interaction. Here an account of the MAPK substrates found either by one to one *in-vitro*/*in-vivo* assays or on a large scale by using Y2H, BiFC or protein microarray chips is discussed (**Table 2**).

MKS1 and WRKY transcription factors

Both MAPKs and WRKY transcription factors are known to play prominent roles in transducing cellular signaling especially in plant innate immune responses. 'MPK4 substrate 1' (MKS1) was originally identified as AtMPK4 interacting partner in a yeast two-hybrid screen by Andreasson *et al.* (2005) and a subsequent screen using MKS1 as bait detected interactions with two transcription factors, WRKY25 and WRKY33. These interactions have been validated by co-immunoprecipitation experiments and are consistent with transcript analyses of whole transgenic plants. Interestingly, challenge with flg22, a flagellin-derived peptide, leads to activation of AtMPK4 which leads to nuclear dissociation of an AtMPK4–MKS1–WRKY33 complex and release of WRKY33 and MKS1. WRKY33 then activates the expression of PAD3 (phytoalexin deficient 3). PAD3 is required for the synthesis of the antimicrobial camalexin (Qiu *et al.* 2008). By contrast, MKS1 and WRKY33 are associated *in-planta* both before and after pathogen challenge. MKS1 is the only AtMPK4 substrate identified to date. Rigorous demonstration that WRKY33 is an *in-vivo* AtMPK4 substrate has yet to be reported. But in contrast, Mao *et al*. (2011) provide strong *in-vitro* and *in-vivo* evidences that AtMPK3 and AtMPK6 directly phosphorylate the transcription factor WRKY33 during *Botrytis cinerea* infection, which leads to the long-term camalexin biosynthesis. Moreover, WRKY33 directly binds to its own promoter for autoregulation and to the promoter of PAD3, one of the camalexin biosynthesis genes.

Arabidopsis MEKK1 has been shown to interact directly with and phosphorylate the WRKY53 transcription factor, as well as binding directly to its promoter and play a role in leaf senescence (Miao *et al.* 2007). Transient expression analysis and bimolecular fluorescence complementation assay proved the MEKK1- WRKY53 interaction.

In a protein microarray approach WRKY53 has also been found to serve as an *in-vivo* substrate for several MAPKs (e.g. AtMPK6, AtMPK7 and AtMPK10) (Popescu *et al.* 2009) but the functional consequences of these phosphorylation events, and how they might be integrated with possible activation by MEKK1, remain to be established. *Arabidopsis* WRKY22 and WRKY29 transcription factors were earlier implicated as operating downstream of AtMPK3 and AtMPK6 (Asai *et al.* 2002). Also flg22 activated AtMAPK pathway activates WRKY28 helping in enhancing salicylic acid (SA) production via the ICS1 expression (Navarro *et al.* 2004).

By using a targeted Y2H screen, the tobacco transcription factor WRKY1 has been shown as a potential substrate of SIPK, a MAPK of tobacco (Menke *et al.* 2005). *Nicotiana benthamiana* WRKY8 transcription factor was shown as a physiological substrate of SIPK, NTF4, and WIPK. The interaction of WRKY8 with MAPKs depended on its D domain, which is a MAPK interacting motif, and this interaction was required for effective phosphorylation of WRKY8 in plants. MAPK mediated phosphorylation of WRKY8 has an important role in the defense response through activation of downstream genes (Ishihama *et al.* 2011). Recently, Son *et al.* (2012) made an attempt to decipher whole chitin-induced defence signaling network using a large scale Y2H screen. Besides finding some important ERFs, they could find five WRKY factors namely WRKY6, 18, 33, 40 and 72 working in a close signaling complex with AtMPK3 and AtMPK6 to induce an efficient innate immune response.

VIP1

The *Arabidopsis* bZIP transcription factor VIP1 (VirE1-INTERACTING PROTEIN 1) is involved in nuclear import of the VirE2/T-DNA complex during *Agrobacterium*-plant transformation (Tzfira *et al.* 2001). In both yeast two-hybrid screens and *in-planta* co-immunoprecipitation techniques, AtMPK3 was found to interact with VIP1 (Djamei *et al.* 2007). VIP1 can be phosphorylated *in-vitro* by AtMPK3 at Ser79 (a low stringency SP motif) when induced by flg22 or *Agrobacterium* inoculation. Ser79 function was probed by using phospho-mimic (VIP1D) and nonphosphorylatable (VIP1A) mutant forms. This phosphorylation leads to VIP1 translocation from the cytosol to the nucleus within 5 minutes of phosphorylation thereby enhancing the transformation efficiency of *Agrobacterium*. While *Agrobacterium* exploits AtMPK3 phosphorylated VIP1 to deliver T-DNA to host nuclei, on the other hand VIP1 directly binds to VIP1 responsive elements (VRE) and enhances MYB44 and TRXH8 expression within 10–20 min after flg22 stimulation (Pitzschke *et al.* 2009). VIP1 may heterodimerize with other TFs to control more primary defense genes. VIP1 also binds to PR1 (PATHOGENESIS RELATED1) promoter indirectly activating the transcription of late stress-responsive genes.

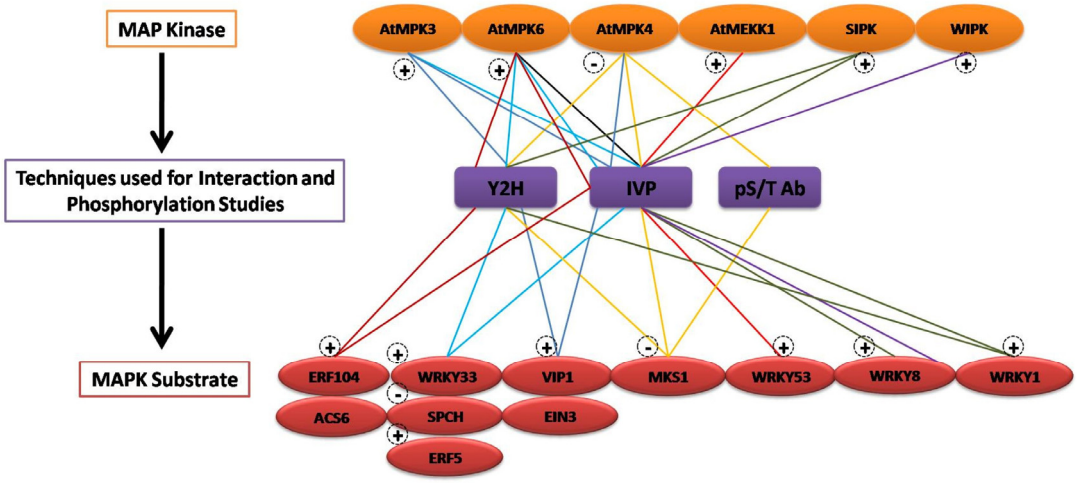

Fig. 1 Downstream MAPK substrates identified and characterized by Y2H (yeast two-hybrid), IVP (*in-vitro* phosphorylation) and or pS/T Ab (phospho serine/threonine antibodies). The same line color shows the link between MAPK and downstream substrate. For simplicity, substrates sharing the same MAPK activator are shown together. The positive physiological effect of the interaction is shown by + sign while negative effect is shown by – sign. AtMPK3 positively regulates ethylene biosynthesis and signaling by interacting and phosphorylating the EIN3 (Yoo *et al*. 2008), ERF5 (Son *et al*. 2012) while AtMPK6 phosphorylates ERF104 (Bethke *et al*. 2009) and ACS6 (Liu and Zhang 2004). AtMPK3 and AtMPK6 by interacting and phosphorylating WRKY33 act as positive regulators of plant innate immunity (Mao *et al*. 2011). The phosphorylated of SPCH by AtMPK3 and AtMPK6 imparts a negative control on stomatal development (Lampard *et al*. 2008). AtMPK3 phosphorylated VIP1 is used to deliver *Agrobacterium* T-DNA to host nuclei and also induce immune response (Djamei *et al*. 2007). AtMPK4 interacts and phosphorylate MKS1 causing its release from WRKY33 which then positively regulates plant defense (Andreasson *et al*. 2005). AtMEKK1 by directly phosphorylating WRKY53 play a role in leaf senescence (Miao *et al*. 2007). SIPK and WIPK mediated phosphorylation of WRKY8 has an important role in the tobacco defense response through activation of downstream genes (Ishihama *et al.* 2011).

bHLH SPEECHLESS

It was already known that AtMPK3 and AtMPK6 are involved in control of stomatal patterning via the multifunctional MEKK YODA-AtMKK4/5-AtMPK3/6 cascade(s). Recently, the downstream component of this pathway was found to be a basic helix–loop–helix (bHLH) transcription factor namely SPEECHLESS (SPCH) (Lampard*et al.* 2008). Unphosphorylated SPCH normally acts as a positive regulator of stomatal development by possibly regulating the expression of the bHLH factor SCREAM (SCRM) (Kanaoka *et al.* 2008). SPCH was shown to be an *in-vitro* and *in-vivo* phosphorylation substrate for both AtMPK3 and AtMPK6. The phosphorylation leads to SPCH degradation and represses its effects on stomatal development. All the five phosphorylation sites investigated had the PXS/TP consensus motif. Interestingly, deletion of these sites in SPCH led to stomatal overproduction, indicating that their phosphorylation is required to repress SPCH activity. However, there was also evidence that phosphorylation of one specific site (Ser193) had a positive effect on SPCH activity, indicating that differences in the pattern of phosphorylation events might potentially fine tune this protein. The other supportive evidence showed that the *mpk3* and *mpk6* loss-of-function mutants resulted in stomatal overproduction and the loss-of-function *spch* mutant could not produce any stomata. In nutshell, MAPK phosphorylation acts to negatively regulate SPCH function, most likely because of changes in SPCH stability.

Ethylene signaling and biosynthesis components

The involvement of MAPK pathway in ethylene signaling and biosynthesis has been a point of controversy ever since the identification of CTR1, a Raf like MAPKKK an important negative regulator of the ethylene signaling pathway (Keiber *et al.* 1993). Since the scope of this article does not allow us to explore the MAPK-ethylene signaling in detail, we will focus only on components of ethylene signaling and biosynthesis identified as MAPK substrate. Two transcription factors, EIN3 (ethylene insensitive) and ERF104 (ethylene response factor) and a rate limiting enzyme in ethylene biosynthesis ACS (1-aminocyclopropane-1-carboxylate synthase) have so far been shown to interact directly with MAPKs. Arabidopsis AtMPK3 and AtMPK4 were shown to target EIN3, in an *in-vitro* phosphorylation assay and also in a protoplast based assay system using co-immunoprecipitation (Yoo *et al.* 2008). Phosphorylation of EIN3 by AtMPK6 affects stability of the protein. In an another study using yeast two-hybrid screen Bethke *et al.* (2009) showed interaction of AtMPK6 with ERF104. This result was validated using fluorescence resonance energy transfer (FRET). Interestingly, the recombinant transcription factor was specifically phosphorylated by AtMPK6 and not by AtMPK3. The phosphorylation of ERF104 by AtMPK6 has its biological relevance against pathogen attack. Phosphorylation of ACS6, the rate limiting enzyme in ethylene biosynthesis by AtMPK6 was shown by Liu and Zhang (2004). The phosphorylation of ACS6 was shown in *in-vitro* phosphorylation assay using activated AtMPK6 and also in an in-gel kinase assay using ACS6 as substrate. The phosphorylation of ACS6 leads to the stabilization and accumulation of the protein. Later it was shown that unphosphorylated ACS6 undergoes rapid degradation by the 26S proteasome pathway (Joo *et al.* 2008).

In conclusion, the plant MAPK substrates identified so far using either *in-vitro* or *in-vivo* techniques are summarized in **Fig. 1**.

FUTURE PERSPECTIVE

Lack of in-depth information about the actual physiological substrate of MAPK in plants is hampering our progress in understanding this crucial signaling cascade. Though we have some information about the targets of some of the MAPKs upon activation, still comprehensive knowledge about the substrate needs to be generated. Most of our current information is from model eudicot plant, *Arabidopsis* and a few from tobacco. Interestingly there is no information of MAPK substrate from model monocot plant, rice. Out of all the techniques used to decipher the targets, protein microarray provided maximum number of putative candidates. Validation of most of the putative candidate as MAPK substrate and its biological significance is still awaited. A more concentrated effort to establish protein microarray system from different economically important

crop is required. Additionally, the information generated in *Arabidopsis* needs to be extrapolated in other plant systems.

ACKNOWLEDGEMENTS

Authors acknowledge Department of Biotechnology and core grant of NIPGR for financial support. AHS thanks Council of Scientific and Industrial Research, India (CSIR Award No: 09/803(0066)/2009-EMR-I) and HA thanks Department of Science and Technology, India (DST award No. SR/WOS-A/LS-158/2010) for a fellowship.

REFERENCES

Andreasson E, Jenkins T, Brodersen P, Thorgrimsen S, Petersen NH, Zhu S, Qiu JL, Micheelsen P, Rocher A, Petersen M, Newman MA, Bjørn Nielsen H, Hirt H, Somssich I, Mattsson O, Mundy J (2005) The MAP kinase substrate MKS1 is a regulator of plant defense responses. *EMBO Journal* **24**, 2579-2589

Asai T, Tena G, Plotnikova J, Willmann MR, Chiu WL, Gomez-Gomez L, Boller T, Ausubel FM, Sheen J (2002) MAP kinase signaling cascade in *Arabidopsis* innate immunity. *Nature* **415**, 977-983

Banko MR, Allen JJ, Schaffer BE, Wilker EW, Tsou P, White JL, Villén J, Wang B, Kim SR, Sakamoto K, Gygi SP, Cantley LC, Yaffe MB, Shokat KM, Brunet A (2011) Chemical genetic screen for AMPKα2 substrates uncovers a network of proteins involved in mitosis. *Molecular Cell* **44**, 878-892

Berwick DC, Tavare JM (2004) Identifying protein kinase substrates: Hunting for the organ-grinder's monkeys. *Trends in Biochemical Sciences* **29**, 227-232

Bethke G, Unthan T, Uhrig JF, Poschl Y, Gust AA, Scheel D, Lee J (2009) Flg22 regulates the release of an ethylene response factor substrate from MAPK kinase 6 in *Arabidopsis thaliana* via ethylene signaling. *Proceedings of the National Academy of Sciences USA* **106**, 8067-8072

Boehmer M, Romeis T (2007) A chemical-genetic approach to elucidate kinase function in planta. *Plant Molecular Biology* **65**, 817-827

Djamei A, Pitzschke A, Nakagami H, Rajh I, Hirt H (2007) Trojan horse strategy in *Agrobacterium* transformation: Abusing MAPK defense signaling. *Science* **318**, 453-456

Feilner T, Hultschig C, Lee J, Meyer S, Immink RG, Koenig A, Possling A, Seitz H, Beveridge A, Scheel D, Cahill DJ, Lehrach H, Kreutzberger J, Kersten B (2005) High throughput identification of potential Arabidopsis mitogen-activated protein kinases substrates. *Molecular and Cellular Proteomics* **4**, 1558-1568

Ishihama N, Yamada R, Yoshioka M, Katou S, Yoshioka H (2011) Phosphorylation of the *Nicotiana benthamiana* WRKY8 transcription factor by MAPK functions in the defense response. *Plant Cell* **23**, 1153-1170

Joo S, Liu Y, Leuth H, Zhang S (2008) MAPK phosphorylation induced stabilization of ACS6 protein is mediated by the non-catalytic C-terminal domain, which also contains the *cis*-determinant for rapid degradation by the 26S proteasome pathway. *Plant Journal* **54**, 129-140

Kanaoka MM, Pillitteri LJ, Fujii H, Yoshida Y, Bogenschutz NL, Takabayashi J, Zhu JK, Torii KU (2008) SCREAM/ICE1 and SCREAM2 specify three cell-state transitional steps leading to *Arabidopsis* stomatal differentiation. *Plant Cell* **20**, 1775-1785

Kersten B, Agrawal GK, Durek P, Neigenfind J, Schulze W, Walther D, Rakwal R (2009) Plant phosphoproteomics: An update. *Proteomics* **9**, 964-988

Kieber JJ, Rothenberg M, Roman G, Feldmann KA, Ecker JR (1993) CTR1, a negative regulator of the ethylene response pathway in *Arabidopis*, encodes a member of the raf family of protein kinases. *Cell* **72**, 427-441

Kramer A, Feilner T, Possling A, Radchuk V, Weschke W, Bürkle L, Kersten B (2004) Identification of barley CK2 alpha targets by using the protein microarray technology. *Phytochemistry* **65**, 1777-1784

Lampard GR, Macalister CA, Bergmann DC (2008) *Arabidopsis* stomatal initiation is controlled by MAPK-mediated regulation of the bHLH SPEECHLESS. *Science* **322**, 1113-1116

Lee JS, Huh KW, Bhargava A, Ellis BE (2008) Comprehensive analysis of protein–protein interactions between *Arabidopsis* MAPKs and MAPK kinases helps define potential MAPK signalling modules. *Plant Signaling and Behavior* **3**, 1037-1041

Liu Y, Zhang S (2004) Phosphorylation of 1-aminocyclopropane-1-carboxylic acid synthase by MPK6, a stress-responsive mitogen activated protein kinase, induce ethylene biosynthesis in *Arabidopis*. *Plant Cell* **16**, 3386-3399

Mao G, Meng X, Liu Y, Zheng Z, Chen Z, Zhang S (2011) Phosphorylation of a WRKY transcription factor by two pathogen-responsive MAPKs drives phytoalexin biosynthesis in *Arabidopsis*. *Plant Cell* **23**, 1639-1653

Martin K, Steinberg TH, Cooley LA, Gee KR, Beechem JM, Patton WF (2003) Quantitative analysis of protein phosphorylation status and protein kinase activity on microarrays using a novel fluorescent phosphorylation sensor dye. *Proteomics* **3**, 1244-1255

Menke FL, Kang HG, Chen Z, Park JM, Kumar D, Klessig DF (2005) Tobacco transcription factor WRKY1 is phosphorylated by the MAP kinase SIPK and mediates HR-like cell death in tobacco. *Molecular Plant-Microbe Interactions* **18**, 1027-1034

Miao Y, Laun TM, Smykowski A, Zentgraf U (2007) *Arabidopsis* MEKK1 can take a short cut: it can directly interact with senescence-related WRKY53 transcription factor on the protein level and can bind to its promoter. *Plant Molecular Biology* **65**, 63-76

Navarro L, Zipfel C, Rowland O, Keller I, Robatzek S, Boller T, Jones JDG (2004) The transcriptional innate immune response to flg22 interplay and overlap with Avr gene-dependent defense responses and bacterial pathogenesis. *Plant Physiology* **135**, 1113-1128

Pitzschke A, Djamei A, Teige M, Hirt H (2009) VIP1 response elements mediate mitogen-activated protein kinase 3-induced stress gene expression. *Proceedings of the National Academy of Sciences USA* **106**, 18414-18419

Popescu SC, Popescu GV, Bachan S, Zhang Z, Gerstein M, Snyder M, Dinesh-Kumar SP (2009) MAPK target networks in *Arabidopsis thaliana* revealed using functional protein microarrays. *Genes and Development* **23**, 80-92

Ptacek J, Devgan G, Michaud G, Zhu H, Zhu X, Fasolo J, Guo H, Jona G, Breitkreutz A, Sopko R, McCartney RR, Schmidt MC, Rachidi N, Lee SJ, Mah AS, Meng L, Stark MJ, Stern DF, De Virgilio C, Tyers M, Andrews B, Gerstein M, Schweitzer B, Predki PF, Snyder M (2005) Global analysis of protein phosphorylation in yeast. *Nature* **438**, 679-684

Pusch S, Dissmeyer N, Schnittger A (2011) Bimolecular-fluorescence complementation assay to monitor kinase-substrate interactions *in vivo*. *Methods in Molecular Biology* **779**, 245-257

Qiu JL, Fiil BK, Petersen K, Nielsen HB, Botanga CJ, Thorgrimsen S, Palma K, Suarez-Rodriguez MC, Sandbech-Clausen S, Lichota J, Brodersen P, Grasser KD, Mattsson O, Glazebrook J, Mundy J, Petersen M (2008) *Arabidopsis* MAP kinase 4 regulates gene expression through transcription factor release in the nucleus. *EMBO Journal* **27**, 2214-2221

Rodriguez MC, Petersen M, Mundy J (2010) Mitogen-activated protein kinase signaling in plants. *Annual Review of Plant Biology* **61**, 621-649

Sinha AK, Jaggi M, Raghuram B, Tuteja N (2011) Mitogen activated protein kinase signaling in plants under abiotic stress. *Plant Signaling and Behavior* **6**, 196-203

Sharrocks AD, Yang SH, Galanis A (2000) Docking domains and substrate-specificity determination for MAP kinases. *Trends in Biochemical Sciences* **25**, 448-453

Son GH, Wan J, Kim HJ, Nguyen XC, Chung WS, Hong JC, Stacey G (2012) The ethylene responsive element binding factor 5, ERF5, is involved in the chitin-induced innate immunity response. *Molecular Plant-Microbe Interactions* **25**, 48-60

Tzfira T, Vaidya M, Citovsky V (2001) VIP1, an *Arabidopsis* protein that interacts with *Agrobacterium* VirE2, is involved in VirE2 nuclear import and *Agrobacterium* infectivity. *EMBO Journal* **20**, 3596-3607

Vlad F, Turk BE, Peynot P, Leung J, Merlot S (2008) A versatile strategy to define the phosphorylation preferences of plant protein kinases and screen for putative substrates. *Plant Journal* **55**, 104-117

Yoo SD, Cho YH, Tena G, Xiong Y, Sheen J (2008) Dual control of nuclear EIN3by bifurcate MAPK cascades in C_2H_4 signaling. *Nature* **451**, 689-795