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

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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 MAPKKKs. MAPKKKs activate downstream MAPKs through phosphorylation on two serine/threonine residues in a conserved S/T–X3–5–S/T motif. On the other hand MAPKs 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 includes the broad experimental manipulation of these 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 (MAPKK), 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 nuclear 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.

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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 MAPKKKs. MAPKKKs activate downstream MAPKs through phosphorylation on two serine/threonine residues in a conserved S/T–X3–5–S/T motif. On the other hand MAPKs 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-
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:

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 MgCl₂ and [γ-32P]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 MnCl₂ in place of MgCl₂ and radio-labelled ATP. Potential substrates are detected by autoradiography of the microarrays.

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 MgCl₂ and radio-labelled ATP. Potential substrates are detected by autoradiography of the microarrays.

3. **In-vivo assays**

   Antibody based method where proteins phosphorylated in the consensus phosphorylation site by the kinase can be detected by western analysis. Here two types of antibodies namely phosphospecific and phospho-motif specific antibodies are used.

**Table 1** Approaches employed for plant MAPK substrate identification.

<table>
<thead>
<tr>
<th>Technique</th>
<th>Methodology</th>
<th>Pros and Cons</th>
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</table>
| 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-32P]ATP. The possible substrates can be shortlisted on the basis of yeast two-hybrid (Y2H) or bi-molecular fluorescence complementation (BiFC) interaction screening methods. 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 MgCl₂ and radio-labelled ATP. Potential substrates are detected by autoradiography of the microarrays. | Pros: Useful and easy method  
Cons: Random fishing approach, low throughput, high background noise. |
| 2. Protein microarrays         | Chip-based method wherein the possible MAPK substrate proteins are translated either *in-vitro* or *in-planta* and blotted on the chips. The chips are then incubated with purified MAPKs in the presence of kinase buffer containing MgCl₂ 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 wherein proteins phosphorylated in the consensus phosphorylation site by the kinase can be detected by western 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. |

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 MgCl₂ 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 FAST²⁹ 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 to probe high-density protein-phosphorylation arrays 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, defense, and stress responses. Transient in-planta activation of some of these transcription factors (TGA1, WRKY6, WRKY8, tRNA-RRY53, 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 relationships 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 multwell plates and incubated in solution with the kinase of interest to allow the immobilization of the phosphorylated peptides on the same site as the kinase. This approach is a faster, more sensitive, and 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, certain kinases expressed in planta 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-vitro 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/T]P 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 PxxS/P site will be phosphorylated by a MAPK or not. Andreason et al. (2000) found that 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 system.
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.

<table>
<thead>
<tr>
<th>MAPK</th>
<th>Substrate</th>
<th>Plan system</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>AtMPK4</td>
<td>MKS1</td>
<td>Arabidopsis</td>
<td>Andreaasson et al. 2005</td>
</tr>
<tr>
<td>AtMPK3, AtMPK6</td>
<td>WRKY33</td>
<td>Arabidopsis</td>
<td>Miao et al. 2011</td>
</tr>
<tr>
<td>AtMEKK1</td>
<td>WRKY53</td>
<td>Arabidopsis</td>
<td>Miao et al. 2007</td>
</tr>
<tr>
<td>10 AtMPKs</td>
<td>570 substrates</td>
<td>Arabidopsis</td>
<td>Poppescu et al. 2009*</td>
</tr>
<tr>
<td>AtMPK3</td>
<td>48 substrates</td>
<td>Arabidopsis</td>
<td>Feilner et al. 2005*</td>
</tr>
<tr>
<td>AtMPK6</td>
<td>39 substrates</td>
<td>Arabidopsis</td>
<td>Feilner et al. 2005*</td>
</tr>
<tr>
<td>AtMPK3, AtMPK6</td>
<td>WRKY22, WRKY29</td>
<td>Arabidopsis</td>
<td>Asai et al. 2002</td>
</tr>
<tr>
<td>SIPK</td>
<td>WRKY1</td>
<td>Tobacco</td>
<td>Menke et al. 2005</td>
</tr>
<tr>
<td>SIPK, NTF4, WIPK</td>
<td>WRKY8</td>
<td>Tobacco</td>
<td>Ishihama et al. 2011</td>
</tr>
<tr>
<td>AtMPK3</td>
<td>VIP1</td>
<td>Arabidopsis</td>
<td>Djamei et al. 2007</td>
</tr>
<tr>
<td>AtMPK3, AtMPK6</td>
<td>SPCH</td>
<td>Arabidopsis</td>
<td>Lampard et al. 2008</td>
</tr>
<tr>
<td>AtMPK3, AtMPK6</td>
<td>ERF5</td>
<td>Arabidopsis</td>
<td>Son et al. 2012</td>
</tr>
<tr>
<td>AtMPK3, AtMPK4</td>
<td>EIN3</td>
<td>Arabidopsis</td>
<td>Yoo et al. 2008</td>
</tr>
<tr>
<td>AtMPK6</td>
<td>ERF104</td>
<td>Arabidopsis</td>
<td>Bethke et al. 2009</td>
</tr>
<tr>
<td>AtMPK6</td>
<td>AC86</td>
<td>Arabidopsis</td>
<td>Liu and Zhang 2004</td>
</tr>
</tbody>
</table>

*Poppescu 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 late stress-responsive 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-vitro 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 AtMPK 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 (Tzhr 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 PRI (Pathogenesis related 1) promoter indirectly activating the transcription of late stress-responsive genes.
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) (Kanakoka 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 PXST/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 mk6 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 (Keibet 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 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.

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