In-silico Approaches for Studying Cross-talk of Different Kinases Associated in Diverse Biological Processes with their Interacting Substrates Partners

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Abstract

The Signal transduction pathway uses docking interaction with kinases which may also be regulated by phosphorylation. Regulation of these docking interactions by phosphorylation allows an additional level of control over the diverse biological processes including Crosstalk induction of defense. However, the present study, attempts have been made to identify the presence of important determinants/motifs for substrate specificity of Mitogen activated protein kinase (MAPK), CaM kinase II, Protein kinase C, Receptor tyrosine kinase in the 54 identified MAPK 3 and MAPK 6 substrates of Arabidopsis thaliana. All identified substrates do not possess the known (p) S/ (p) T-P phosphorylation sites. Out of 54 substrates, 47 have S/T-P site and 7 are lacking such sites for interaction. Likewise 8 shows XRRX(p)S pattern, 28 shows HYDXRX(p)XX pattern , 33 shows HYDXRXRX(p)S pattern, 12 shows R/K(1-3)X(p) S/T(HYD)R/K(1-3) pattern and 17 shows Na(1-3)-X-(p)Y-XX-HYD pattern. In order to ascertain the role of surrounding hydrophobic amino acids in the interaction, the other conserved pattern/Motif were also identified in the MAPK substrates which include XRRX(p)S, HYDXRXXXS, HYDXRXRXS, R/K(1-3)X(p)S/T(HYD)R/K(1-3), Na(1-3)-X-(p)Y-XX-HYD (Na-Hydrophilic; HYD- Hydrophobic; X- Any amino acid) pattern. These conserved patterns show activation sites for other kinases viz. MAPK Activated Protein kinase-1, MAPK Activated Protein kinase -2, CaM Kinase II, Protein kinase C, Tyrosine protein kinase respectively. Identification of interacting partners based on the surrounding amino acids of phosphorylation sites could be useful in the understanding of such complex hierarchical networks involved in controlling the defense signaling pathways.

Keywords: MAPK3 and MAPK6 Substrate; Kinases; Phosphorylation site; Conserved pattern; Homology; Crosstalk

Introduction

Phosphorylation is catalyzed by protein kinases (pks) and activity of these kinases are associated with many biological processes such as development, cell division, cell death, response towards biotic and abiotic stimuli [1]. Considering these reasons, it is important for better understanding how protein kinase select and recognize their interacting partner. Numerous researches have been performed to elucidate the intrinsic mechanisms of phosphorylation in many life phenomena such as cell cycle [2,3]. Protein phosphorylation can occur on serine, threonine and tyrosine residues, as well as histidine and aspartate residues in the case of two-component phosphorelays [4]. Kinases and phosphatase both recognize their substrates through different patterns, or motifs, present near the phosphorylation site in the amino acid sequence of the substrate [5,6].

Phosphorylation usually results in a functional change of the target protein (substrate) by changing enzyme activity, cellular location, or association with other proteins. There is more than 500 protein kinase [7] and ATP binding site in kinases is highly conserved. The three-dimensional structures of several protein kinases, some with bound substrates and nucleotides, have been determined [8]. All protein kinases show a common fold, consisting of two lobes hinged through a short linker region. The extra cellular domain serves as the ligand-binding part of the molecule. The intracellular or cytoplasmic domain is responsible for the (highly conserved) kinase activity, as well as several regulatory functions [9].

The numbers of known protein kinases have increased at an ever-accelerating pace, it has become more challenging to determine which protein kinases interact with which substrates in the cell and also which kinase interact with other kinase. Likewise there are some Kinases which have mixed kinase activities, like MAP Kinase Kinase (MAPKK) dual specific kinase is involved in the MAP kinase cascade, are a mixed serine/threonine and tyrosine kinase. Some of the kinase deal within this study are Mitogen Activated protein kinase(MAPK), Protein kinase C(PKC), Ca/Calmodulin dependent kinase II(CaM Kinase II), Tyrosine protein kinase, MAPK Activated protein kinase-1 & MAPK Activated protein kinase -2.

The Protein kinase C also known as PKC is a family of enzymes which is involved in controlling the function of other proteins through the phosphorylation of hydroxyl groups of serine and threonine amino acid residues on these proteins. PKC enzymes play an important role in several signal transduction cascades. Beside this, Ca++/calmodulin-dependent protein kinases II or CaM kinases II are serine/threonine-specific protein kinases which are primarily regulated by the Ca++/calmodulin complex and reveals a memory effect on activation, likewise, Tyrosine kinase protein play role in disease resistance signaling. The Receptor Tyrosine Kinase family is present more abundantly in animal then in plant [10]. By searching for Tyr-kinase-specific sequence motifs, several dual specific kinases (DSKs) are identified but no true tyrosine-specific kinases are found in plant [11]. MAPK activated proteins kinase is well known in animal system rather in plant, get

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activated by MAPK, and regulates cell growth and survival in response to hormonal signals [12].

The Mitogen activated protein Kinases (MAPKs) a class of protein kinase shown to play pivotal roles in eukaryotic systems establishes a cognition between sensors and the intrinsic responses, and leads to changes in cellular organization or altered gene expression [13]. A surprisingly large number of genes encoding MAPK pathway components have been uncovered by analyzing model plant genomes. Among them MAPK3 and MAPK 6 are best characterized and are closely related proteins which shows high level of functional redundancy. They play an important role in the development of induced resistance to biotic and abiotic stress in plants [13] and also act as a positive intercessor of defense responses [14]. Their key role in plant growth and development has already been explained.

Until recently it was viewed that signal transduction work as cascade (Simple chain of consecutive states). However, recent research has focused on the divergence, crosstalk, and redundancy between signaling pathways [15,16]. The known MAPK substrate

| S.No | Accession number | Gene Code | Function | Substrate of MAPK3/ MAPK6 |
|------|-----------------|-----------|----------|--------------------------|
| 1    | AY091243        | AT5G23200 | Unknown protein | MAPK3                   |
| 2    | BT001177        | AT2G18020 | 60s ribosomal protein I2 | BOTH                    |
| 3    | BT002063        | AT5G58620 | Putative protein | BOTH                    |
| 4    | BT024222        | AT2G240510 | 40s ribosomal protein s26 | BOTH                    |
| 5    | BT025612        | AT5G02560 | Unknown protein | MAPK3                   |
| 6    | NM_102437       | AT1G26740 | Structural constituent of ribosome | MAPK3 |
| 7    | BT008538        | AT1G77450 | Grab-1-like protein | BOTH                    |
| 8    | BT006316        | AT1G02840 | Ribonucleoprotein s-2-like protein | BOTH |
| 9    | BT026509        | AT3G11510 | Putative 40s ribosomal protein s14 | BOTH |
| 10   | BT002067        | AT5G19290 | Phospholipase-like protein | BOTH |
has also been studied to canvas the crosstalk of different kinases. Conventional experimental methods that measure kinase-substrate interaction require some hypothesis as to the kinase involved in the phosphorylation. Computational methods to predict kinase-substrate interactions from structural information [9] or from other known kinase substrates [17] have also been introduced, but such additional information is not completely available.

Materials and Methods

Phosphorylation site prediction of Known substrate of MAPK3 and MAPK6, Arabidopsis [18] (Table 1) is done by using NetPhos 2.0 server [17] and then nearby sequence of Phosphorylation site was studied to find out the consensus sequences or motif which is phosphorylated by other kinases.

The sequences/motif which is searched for this study is SP/TP for MAPK, HYDXXRXXS for CaM Kinase II,R/K(p)X(T/H)YR/K, for PKC and (Na)-1-3-X-Y-XX-HYD for Receptor tyrosine kinase, HYDXXRXXS for MAPKAP Kinase-2 and XKRXXSXX for MAPKAP Kinase-1 (X- Any amino acid, Na-hydrophilic, HYD-Hydrophobic).

Results

After the study of Arabidopsis thaliana genome it can be concluded that the plants have acquired signal transduction pathway component different from animals as Tyrosine phosphorylation is less common in plants because plant genome do not encode for such receptor tyrosine kinase, where as animal contain a large family of receptor tyrosine kinases. Due to this reason the study emphasized more on Serine-Threonine phosphorylation site then on tyrosine site.

Phosphorylation site prediction of Known substrate of MAPK3 and MAPK6, Arabidopsis is done by using NetPhos 2.0 server (http://www.cbs.dtu.dk/services/NetPhos/). NetPhos results shows that a single phosphorylation site might be recognized by other kinases.

Table 3: Predicting sequences having (p)TP site, but lacking (p)SP site.

| Gene code | Pattern | Gene code | Pattern |
|-----------|---------|-----------|---------|
| AT2G40510 | RRRVPPTPPR RREDTPKPG | AT2G39460 | KISATPRNK YVRLTP KDYD |
| AT3G49830 | LGFKTPREA | AT5G48760 | EGVPPTYDK |
| AT5G45775 | LSQGTPVFS | AT5G10360 | QGVLTPGVR LHRTFCFRLQ TVLTL |
| AT3G58700 | LSQGTPVFS | AT5G14320 | PASCINNKQ |
| AT4G47570 | KVFFTPRXY | AT3G07110 | EGVPPTYD |
| AT5G22845 | NPYSTPTEV |

Table 3 explains the presence of (p)TP site in all those sequences where (p)SP sites are absent, having threshold value above 0.500 when analyzed by NetPhos 2.0 server.

Table 3: Predicting sequences having (p)TP site, but lacking (p)SP site.

Table 2: Predicting the (p)SP/T-site in all studied sequences.
in that 2 sequences i.e. AT2G18020, AT4G17390 are the substrate for both kinases i.e MAPK 3 and MAPK 6.

Analysis for CaM-Dependent Protein Kinase motif (HYDXXRXS; HYD-Hydrophobic, X-any amino acid)

Calcium signaling is one of the best documented pathway in plants and the best known Ca²⁺ sensor is calmodulin (CaM). The active Ca²⁺/CaM complex interacts with target proteins and regulates their activity [20]. Ca/calmodulin (CaM)-dependent protein kinase (CaM II) is a ubiquitous mediator of Ca²⁺ -linked signaling that phosphorylates a wide range of substrates to co-ordinate and regulates Ca²⁺- mediated alterations in cellular function. The core consensus sequence for CaM II HYDXXRXS(p)S where X is any amino acid; HYD- Hydrophobic amino acid [21]. CaM kinase II, Homo sapiens (Accession number- Q13554), sequence has been retrieved, and from NCBI BLASTp it reveals 42% homology with CPK 14, Calmodulin dependent protein kinase in Arabidopsis thaliana (Accession number NP_973661.1) which play a pivotal role in amplifying and diversifying the action of Ca²⁺-mediated signals [22]. When CaM Kinase II conserved pattern was analyzed in our sequences it was found that out of 54 sequences 35 shows the pattern as shown in Table 5.

Table 6 explains the presence of Conserved pattern for protein kinase C, which shows that MAPK substrate also contain recognition motif for Protein Kinase C.

Analysis for Tyrosine Kinase Protein -Na₁,X₋(p)Y₋XX- HYD(Na-Hydrophilic; HYD-Hydrophobic)

The Intracellular signaling is mediated by association of multi-protein complex where tyrosine phosphorylation initiates downstream signaling by creating sequence-specific recognition sites and phosphotyrosine-binding domains which facilitate the assembly of multi-protein complex [23,24]. Although Tyrosine phosphorylation is less common in plants, a recent study indicates that phosphorylation is less common in plants, a recent study indicates that MAPK substrate also contain recognition motif for CaM Kinase.

Table 5 explains the presence of Conserved pattern for CaM kinase II, which shows that MAPK substrate also contain recognition motif for CaM kinase II.

| Gene code | Pattern | Gene code | Pattern |
|-----------|---------|-----------|---------|
| AT3G52580 | AXRXSG | AT5G66940 | VXRXSG |
| AT2G40510 | VXRXSG | AT5G48760 | FRXXSG |
| AT1G26740 | FXRXSG | AT5Q23200 | VXRXSG |
| AT4G15000 | VXRXSG | AT5G58620 | YXRXSG |
| AT3G48930 | LXRXSG | AT1G02840 | HXRXSG |
| AT1G64370 | GXRXXS | AT5G19290 | HXRXSG |
| AT5G48990 | LXRXXS | AT4G03260 | AXRXSG |
| AT5G10360 | FXRXSG | AT4G39880 | GXRXXS |
| AT1G22160 | GXRXXS | AT5G02560 | AXRXSG |
| AT2G19730 | LXRXXS | AT3G58700 | IRXRXS |
| AT5G62070 | FRXXSG | AT5G45775 | FRXXSG |
| AT5G54630 | MXXRXS | AT5G06140 | IRXRXS |
| AT5G44100 | YXRXSG | AT4G11280 | FXRXSG |
| AT3G07350 | LXRXXS | AT1G23860 | YXRXSG |
| AT5G21100 | MXXRXS | AT5G65360 | AXRXSG |
| AT5G47570 | VXRXSG | AT2G46020 | WXXRXS |
| AT3G04400 | LXRXXS | AT2G18020 | VXRXSG |
| AT3G60390 | IRXRXS | AT1G02840 | HXRXSG |

Table 5: Predicted the CaM Kinase II conserved pattern i.e HYDXXRXS(p)S.

Table 6 explains the presence of Conserved pattern for protein kinase C, which shows that MAPK substrate also contain recognition motif for Protein Kinase C.

| Gene code | Pattern |
|-----------|---------|
| AT4G15000 | XRXSGK |
| AT5G10360 | RXXSRX |
| AT5G44100 | XXTXXK |
| AT3G07350 | XRXXLRX |
| AT1G23860 | XRXXSRX |
| AT4G17390 | XXTXXK |
| AT3G58700 | XRXXSRX |
| AT2G18020 | XRXXFRX |
| AT5G65360 | XXCRXX |
| AT3G58700 | XRXXFRX |
| AT5G54630 | XRXXXK |
| AT5G45775 | XRXXFRX |

Table 6: Predicted the protein kinase C, conserved pattern i.e R/K₁₋₃₋X(p) S/S(T)HYD/R(K₁₋₃₋S).

Table 6: Predicted the protein kinase C, conserved pattern i.e R/K₁₋₃₋X(p) S/S(T)HYD/R(K₁₋₃₋S), conserved patterns i.e Na₁₋₃₋X₋(p)Y₋XX-HYD (Na-hydrophilic; HYD-hydrophobic) was observed in all MAPK substrate that are used in our study, it shows that out of 54, 17 shows tyrosine kinase protein recognition pattern as shown in Table 7.

Analysis for MAPK AP kinase-1 and MAPK AP kinase-2 (XXRXSXSS & HYDXXRXSXX)

Many cellular responses of MAPK cascades have been shown to be mediated by MAP kinase-activated protein kinases (MAPKAPK). The MAPKAP-K family is activated by the ERK and JNK in mammalian system whereas MAPKAP-2 is capable of directly phosphorylating transcription factors [27].

Table 4: Predicting sequences lacking (p)S/TP site.

| Gene code | Pattern |
|-----------|---------|
| AT2G19730 | VXFSKX |
| AT4G15000 | AXRXSV |
| AT5G65360 | AXSTSGG |
| AT2G18020 | AGXSFGG |
| AT3G04400 | FXSMLG |
| AT4G39200 | AXSFGG |
| AT4G17390 | XXXSVER |
| AT3G16640 | FXSMLG |
| AT5G02560 | AXRXSV |

Table 4 shows that 7 sequences out of 54 lack (p)S/TP site.
MAPK AP-1, Homo sapiens (Accession number- Q9BPZ7), sequence has been retrieved and from NCBI BLASTp it reveals 38% homology with mitatricopeptidase (PPR) repeat-containing protein in Arabidopsis thaliana (Accession number- NP_194007.1) which help in restoring fertility to cytoplasmic male-sterile plants [28]. Similarly MAPK AP-2 Homo sapiens (Accession number- P94137) sequence has been retrieved and from NCBI BLASTp it reveals 37% homology with CDPK9 (Calmodulin-like domain protein kinase 9) in Arabidopsis thaliana (Accession number NP_197748) which has been implicated in the regulation of cell cycle and transcription [29].

The conserved pattern for MAPK AP-1 and MAPK AP-2 is XXRRX(p)SXX [30], HYDXRXX(p)XXX, respectively [31]. When this pattern was analyzed in all MAPK substrates sequences which are examined in this study, it depict that 8 protein sequences out of all shows conserved pattern for MAPK AP-1 and 28 shows MAPK AP-2

| Gene code  | Pattern     |
|------------|-------------|
| AT1G23200  | QXXXYX      |
| AT1G02840  | PXYYXXV     |
| AT3G11510  | SXYYXM      |
| AT5G19290  | TXXXYX      |
| AT4G03260  | NXYYYXG     |
| AT5G48990  | SXYYXL      |
| AT5G62070  | SXYYXL      |
| AT3G52580  | SXYYXM      |
| AT5G44100  | QXXXYX      |
| AT3G07350  | PXYYXXL     |
| AT1G23860  | PXYYXXA     |
| AT5G07350  | PXYYXXL     |
| AT4G17390  | SXYYYX      |
| AT2G02820  | SXYYXG      |
| AT5G66940  | SXYYXY      |
| AT2G39460  | PXYYXA      |
| AT5G21100  | NXYYYX      |

Table 7 explains the presence of Conserved pattern for tyrosine kinase protein, shows that MAPK substrate also contain recognition motif for tyrosine kinases protein.

| Gene Code | Pattern     |
|-----------|-------------|
| AT5G23200 | XXRWS-------|
| AT1G02840 | XXRXSEX     |
| AT1G64370 | XXRSEX      |
| AT5G10360 | XXRXSEX     |
| AT1G23860 | XXRXRXS     |
| AT1G22160 | XXRXRXS     |
| AT1G35680 | XXRXRXS     |
| AT5G54630 | XXRXRXS     |

Table 8 explains the presence of Conserved pattern for MAPK AP kinase-1 present in MAPK substrate.

| Gene code  | Pattern     |
|------------|-------------|
| AT1G02840  | VXRXRXS     |
| AT5G19290  | HXRXRXS     |
| AT2G19730  | FXRXRXS     |
| AT4G03260  | MXRXRXS     |
| AT4G39880  | GXRXRXS     |
| AT4G15000  | VXRXRXS     |
| AT3G52580  | AXRXRXS     |
| AT5G48760  | HXRXRXS     |
| AT3G07350  | IXRXRXS     |
| AT5G44100  | TXRXRXS     |
| AT3G04400  | LXRXRXS     |
| AT5G45775  | LRXXRXS     |
| AT5G54630  | TXRXRXS     |

Table 9 explains the presence of Conserved pattern for MAPK AP kinase-2 present in MAPK substrate.

Table 9: Predicted the conserved pattern, HYDXRXX (p)XXX for MAPKAP kinase-2.

The conserved pattern which implies that homologue of MAPK activated protein kinase 2 might also be present in plants so this opens a pathway for further analysis. Table 8 and Table 9 shows sequences which show above conserved pattern.

Discussion

This study is based on the study of consensus sequences for short stretches of primary sequences which is required for phosphorylation by different kinases in the known substrate of MAPK-3 and MAPK-6 and postulated that these substrate might be the substrate/ partner of other kinases. It has been already stated that all known MAPK substrate carrying minimal consensus sequence i.e. (p) S/T [32]. The present study demonstrates that the most of the MAPK substrate that has been studied in our analysis also contain some non S/T (p)-site which clearly indicates that their might also be some other recognition motifs, which are also responsible for MAPK Substrate phosphorylation. In contrast to this, these substrate might also contain recognition motif for some other kinases, like, HYDXRXX(p)S motif is present in CaM Kinase II (Ca2+/Calmodulin dependent Kinase protein) that mediate signal transduction pathway where calcium plays an important role as it play role in Ca signal transduction pathway. It has been already stated that in some plants like tobacco, CaM Kinase II plays an important role in its growth and development [33]. Its homologue also play important role in animals too [34], CaM kinase II shows 42% homology with CPK 14, Calmodulin dependent protein kinase in Arabidopsis thaliana. Another kinase whose conserved pattern was analyzed is Protein Kinase C, which is found to be involved in desensitization in modulating membrane in structural event, and it has also been partially purified in Brassica campestris [35]. Protein kinase C shows 39% homology with S6K2- Serine/Threonine protein kinase 2 in Arabidopsis thaliana. Presence of the consensus sequences in these substrate indicate that their must be a crosstalk between MAP Kinase and Protein kinase C, homologue of plant.

Activity of Tyrosine Kinase protein was also studied as it was used for further analysis. Table 8 and Table 9 shows sequences which show above conserved pattern.
known that is essential for signal transmission as phosphorylation of tyrosine residue modulates enzymatic activity creating binding sites for downstream signaling in molecules. Although Arabidopsis genome does not encode receptor tyrosine kinase unlike animal which indicate that tyrosine phosphorylation occurs less frequently in plants [36]. The presence of the consensus sequences which is recognized by Tyrosine protein kinase in these substrate indicate that their homologue must be present in plants which might show the functional homology as MAPKAP-2 plays role in diseases in animals.

On the bases of these studies it can be hypothesized that these substrates might also act as a substrate for other kinases, this must be a question for further wet lab analysis. Follow-up experiments such as in-vitro verification of phosphorylation site in these substrate for different kinases are essential to evaluate any physiological relevance

Based on this analysis a model is proposed (Figure 2) showing the cross talk between different kinases. There are also some substrate which are unique to MAPK3 i.e. At5g02560, At5g17870, At4g39200, At1g02070 and MAPK 6 i.e At2g46020, At4g11280, At3g22845, At5g06140.

Figure 1: Percentage of conserved pattern seen in all MAPK substrate.

Figure 2: Crosstalk between different kinases, activating common substrate is seen. In a figure Green color reflects CaM Kinase, Orange color reflects Protein kinase C, Blue color reflects Tyrosine protein kinase, Grey color reflects MAPK AP-2, Light blue color reflect MAPK AP-1, Brown color reflects MAPK 3 and red color reflects MAPK 6.
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