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Identification of R2R3- MYB Transcription Factor (AtMYB13) as a Novel Substrate of *Arabidopsis* MPK3 and MPK6

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Abstract: Mitogen-activated protein kinase (MPK) cascades are signal transduction pathways that are highly conserved and widespread in all eukaryotic cells, including yeasts, animals and plants. MPKs play a central role for converting extracellular signals, including environmental stresses, into internal signal transduction and activation of intracellular responses. It is also well documented that plant MPKs are activated by a variety of environmental stimuli including salt, cold, wounding, heat, osmotic shock, heavy metal, UV, drought and pathogen attack. However, so far only a limited number of target molecules have been identified. Here, we report a MYB transcription factor, MYB13 that was identified as a novel substrate of MPKs in *Arabidopsis*. Using pull-down assays, MYB13 was shown to physically interact with MPK6 *in vitro*. MYB13 was phosphorylated by recombinant MPK3 and MPK6 proteins. By site-directed mutagenesis, Thr 71 and Ser138 of MYB13 were identified as the site of MPKs phosphorylation. These results indicated that the MPKs directly phosphorylate MYB13 in *Arabidopsis*.

Keywords: MYB transcription factor, MAPK, phosphorylation.

1. Introduction

Mitogen-activated protein kinase (MPK) cascade, a class of protein kinases has been known to play a pivotal role in eukaryotes including animals, yeasts and plants. They are involved in most cell activities, from cell division to death, including cell differentiation and proliferation, cell growth, as well as environmental stress responses [1-6]. This phosphorylation cascade typically comprises of three consecutively acting protein kinases which form a linear cascade and mediate sequential phosphorylation reactions. The classical view of MPK pathway is as MPK kinase kinase (MPKKK) → MPK kinase (MPKK) → MPK [7, 8]. In a general model, stimulated plasma membrane receptors activate

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MPKKK. Sequential phosphorylations ensue as MPKKK activated downstream MPKK at a conserved S/T-X_{3-5}-S/T motif. Here, MPKK phosphorylates Thr and Tyr residues on the conserved TEY motif at the activation loop of MPK. Finally, MPKs as serine/threonine kinasesare able to phosphorylate a wide range of substrates including other kinases and/or transcription and translation factors, thus regulating many cellular processes in response to the initial stimulus. The deactivation and regulation of MPK activity are mediated by tyrosine and serine/threonine-specific phosphatases. Numerous protein kinases with close sequence similarities to MPKs and other kinases belonging to the MPK cascae have been identified in plants [1, 9].

The MYB family of proteins is large, functionally diverse and represented in all eukaryotes. The functions of MYB proteins have been investigated in numerous plant species such as *Arabidopsis*, maize, rice, petunia, snapdragon, grapevine, poplar and apple, using both genetic and molecular analyses. MYB protein are characterized by a highly conserved DNA-binding domain (MYB domain) composed one to four imperfect amino acid sequence repeats (R) of about 52 amino acids. The largest group of plant MYB factors is R2R3-MYBs, containing two MYB repeats that are most similar to R2 and R3 from c-MYB. This family includes hundreds of members in all the terrestrial plants that have been investigated. Although the MYB domains are conserved within R2R3-MYBs, the C-termini are variable, often containing transcriptional activation or repression domains and conserved serine and threonine residues, which may correspond to post-translational modification sites. Numerous R2R3-MYB proteins have been characterized by genetic approaches and found to be involved in the control of plant-specific processes including primary and secondary metabolism, cell fate and identity, developmental processes and responses to biotic and abiotic stresses [10].

In this study, we present several lines of evidence showing that MYB13 is a substrate for MPK3 and MPK6 *in vitro*. We show that MYB13 physically interacted with MPK3 and MPK6 *in vitro* by pull down assay. MYB13 was phosphorylated by recombinant MPK3, 6. The phosphorylation sites on MYB13 were identified. These results showed that R2R3 MYB13 transcription factor is novel substrate of MPK3 and MPK6 in *Arabidopsis*.

2. Methods

2.1. Expression and purification of recombinant proteins in E.coli

The full-length *MPK3*, *MPK4* and *MPK6* cDNA were subcloned into pQE-30 (Qiagen) expression vector to generate MPK3-His, MPK4-His and MPK6-His, respectively. *MYB13* cDNA were subcloned into pGEX-5X-1 (GE Healthcare) expression vector to generate GST-MYB13. All constructs were expressed in *E.coli* strain BL21 (for GST-fusion protein) or *E.coli* strain M15 (for His-fusion protein). The Histidine (His) and Glutathione S-transferase (GST) fusion proteins expressed in bacteria were induced by 1 mM isopropylthio-β-galactoside at 25°C for 3 h. For protein extraction, cells were collected by centrifugation and then sonicated in a lysis buffer (50 mM Tris-HCl, pH 7.5; 1.37 M NaCl; 27 mM KCl; 2 mM PMSF; 0.1% Triton X-100 for the GST-fusion protein and 50 mM NaH_2PO_4; 300 mM NaCl; 10 mM imidazole; 2 mM PMSF; 0.1% Triton X-100 for the His-fusion protein). The MPKs-His, MYB13-GST, MYB13C-His and MYB13N-His recombinant fusion proteins were purified by Ni-NTA agarose (Qiagen) and Glutathione Sepharose (GE Healthcare), respectively according to the manufacturer's instructions.

2.2. Site-directed mutagenesis

The pGEX-MYB13 construct was used as the template for site-directed mutagenesis with

the QuikChange II site-directed mutagenesis kit (Stratagene), according to the manufacture's instruction. Individual constructs were generated with the following substitutions: GST-MYB13 (T71A and S138A). The mutations were confirmed by nucleotide sequencing before protein expression, and the mutant proteins were produced as described for the original protein.

2.3. Pull-down assay

For GST pull-down, approximately 5 μg of GST-MYB13 was bound to glutathione beads in binding buffer (20 mM Tris-HCl, pH 7.5; 200 mM NaCl; 1% Triton X-100; 0.1 mM EDTA; 0.5 mM DTT) for 2 h at 4°C. The binding reaction was washed three times with the binding buffer. Then 5 μg of His-MPKs recombinant proteins were added and incubated for an additional 2 h at 4°C. The pulled down proteins were eluted by boiling and separated by electrophoresis on 10% SDS-PAGE. Bound protein to GST-MYB13 was detected by Western blotting using an anti-His antibody.

2.4. Kinase assay

The *in vitro* phosphorylation was performed in kinase buffer (25 mM Tris–HCl, pH 7.5, 1 mM DTT, 20 mM $MgCl_2$, 2 mM $MnCl_2$, 50 μM ATP). His-MPK3/6 fusion proteins (1 μg) were mixed with GST (1 μg), Myelin basic protein (MBP) (1 μg), GST-MYB13 (2 μg) in 20 μl of kinase reaction. GST and MBP proteins were used as negative and positive substrates, respectively. The reactions were initiated by adding 1 μCi [γ^{32}P] ATP and incubated at 30°C for 30 min. The reactions were stopped by boiling for 5 min and then loading to 12% SDS-PAGE. Gels were stained with Coomassie Brilliant Blue R-250 and then analyzed by exposure to an autoradiograph film.

3. Results

3.1. MYB13 interacts with MPKs

Using yeast two-hybrid screening, MYB13 was identified as a MPK3, 4, 6 interacting protein [11]. To test whether MYB13 is a genuine target of MPK6, we analyzed *in vitro* interaction between MYB13 and MPK3, 4, 6 by using pull-down assays. GST-MYB13 was immobilized to glutathione beads and then incubated with His-MPK3, 4, 6. Protein bound to the beads was precipitated and analyzed by Western blotting using anti-His antibody. His-MPK6 input served as a positive control. As shown in Figure 1A, MYB13 could pull-down all MPK3, 4, 6 fusion protein but not GST protein. This resul indicated the interaction between MYB13 and MPKs *invitro*.

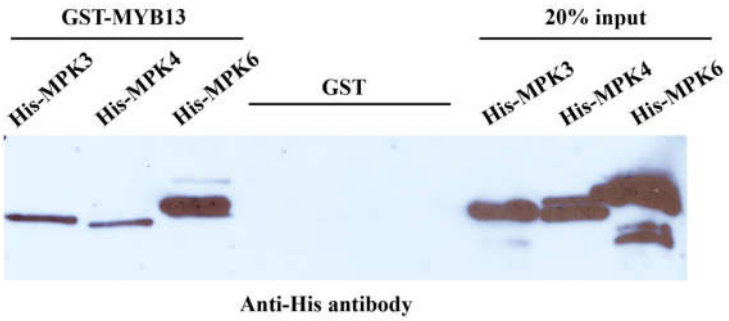

Figure 1. MYB13 physically interacts with MPKs *in vitro*.

The interaction of MYB13 with MPKs in pull-down assay. The equal amount of GST and GST-MYB13 proteins were incubated with glutathione beads, then incubated with His-MPKs in binding buffer. The protein complex was eluted and the association of MYB13 and MPKs was determined by Western blot with the anti-His antibody. 20% input of His-MPK6 (20% input) and purified GST were used as positive and negative controls, respectively.

3.2. MYB13 is phosphorylates by MPK3 and MPK6

To ascertain whether MYB13 is phosphorylated by MPKs, the kinase assay was performed. Purified GST-MYB13 and His-MPK3, His-MPK6 proteins were used for *in vitro* kinase assay. MBP and GST proteins were used as positive and negative controls, respectively. The autophosphorylation activity of His-MPK3 (~43kDa) and His-MPK6 (~46 kDa) were observed. GST-MYB13 (~53 kDa) and MBP (~18.5 kDa) were strongly phosphorylated by MPK6, whereas GST protein (~26 kDa) was not (Fig.2). This result revealed that MPK3 and MPK6 could specifically phosphorylate MYB41 *in vitro.*

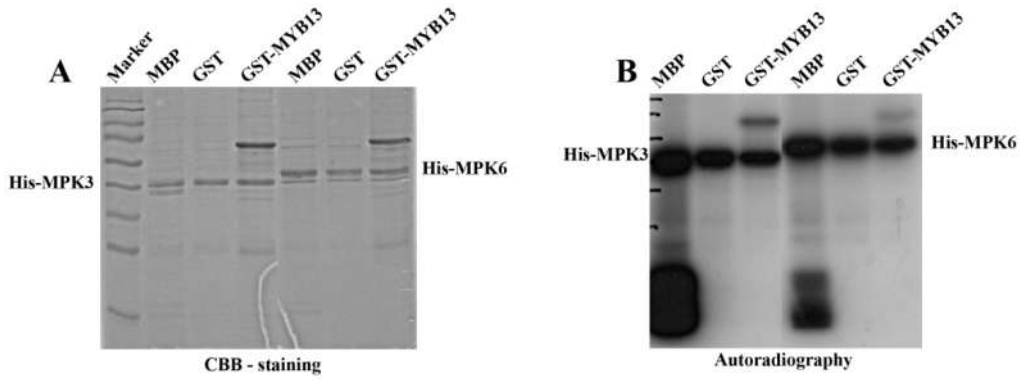

Figure 2. MPK6 phosphorylates MYB13.

In vitro phosphorylation of MYB13 by MPK3 and MPK6. Purified recombinant His-MPK3, His-MPK6 and GST-MYB13 were mixed in kinase reaction buffer and reacted for 30 min at 30°C. The position of molecular weight marker is indicated on the left.

3.3. MYB13 was phosphoryated at Thr^{71} and Ser^{138}

It was documented that the phosphorylation sites of substrates by MPKs are serine or threonine followed by proline (S/T-P motif) [12]. MYB13 contains two potential MPKs phosphorylation sites at Thr^{71} and Ser^{138}. To identify the phosphorylation site of MYB13 by MPKs, we divided MYB13 to two fragments: C- terminal and N- terminal. Both of these fragments were performed to test whether be phosphorylation substrates or not. As shown in figure 3A, the phosphorylated band can be observed in MYB13 N-terminal but not in C-terminal fragment. So, we can conclude that the N-terminal of MYB13 is targeted for phosphorylation. To confirm the phosphorylation sites of MYB13, the site-directed mutagenesis was created. The substitution of Thr^{71} shown reduced phosphorylation signal and the substitution Ser^{138} by Ala could not get the phosphorylation reaction. The double mutant proteins completely abolished the phosphorylation of MYB13 by MPKs. The double mutant was also set up to compare single mutant with wild type protein. The results showed that weak phosphorylation signal was observed in the $MYB13^{T71A}$ mutant protein as well as $MYB13^{S138A}$ and no signal in $MYB13^{T71A/S138A}$ double mutant protein (Fig. 3B). Based on these results, we concluded that Thr^{71} and Ser^{138A} of MYB13 are phosphorylated by MPKs

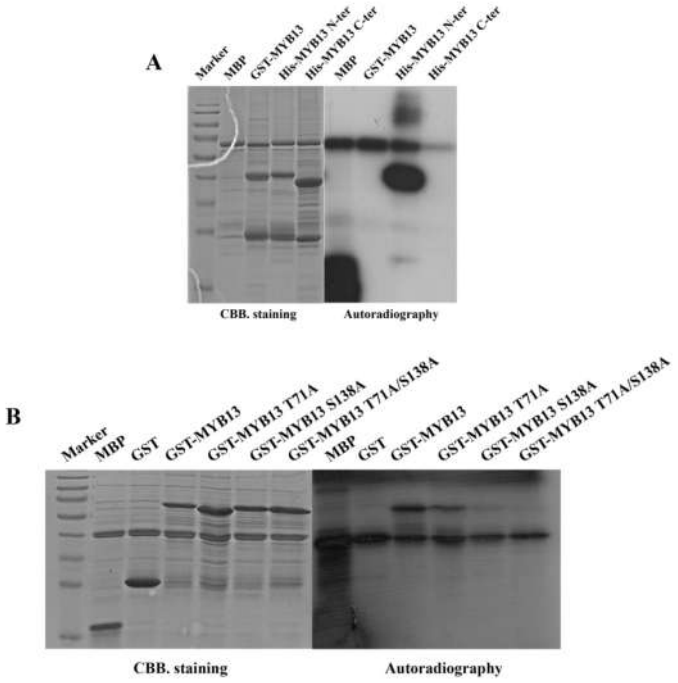

Figure. 3 The phosphorylation sites of MYB13.

(A). The N-terminal fragment of recombinant MYB13 protein contains putative phosphorylation sites.
(B). MYB13 is phosphorylated at Thr^{71} and Ser^{138} by recombinant MPKs.

Kinase reactions were carried out using purified His-tagged MPK3 (MPK3) as enzyme and purified GST, MBP, GST-MYB13, GST-M13YB^{T71A}, GST-MYB13^{S138A}, GST-M13YB$^{T71A/S138A}$, His-MYB13 N-ter and His-MYB13 C-ter as substrates. At the end of the reaction, proteins were resolved on 12% SDS-PAGE. Shown is a gel stained with Coomasie Brilliant Blue (left) and its autoradiograph (right). Protein molecular sizes are shown on the left by arrowheads. The arrowheads on the right indicate position of GST-MYB13, His-MPK3, MBP and GST proteins.

4. Discussion

In eukaryotes, MPK cascades play essential roles in transmitting stimuli from mitogens, developmental cues, and various stresses [13, 14]. In *Arabidopsis*, MPK3, MPK4 and MPK6 are the most extensively studied and are activated by stresses (pathogens, osmotic, cold, and oxidative), developmental cues and auxin signaling [3, 5, 15]. Their multi functionality and signaling specificity are conferred by their ability to phosphorylate different substrates. Several attempts have been made to identify the substrates and interaction partners of MPKs [7]. To date only a limited number of *Arabidopsis* MPK substrates have been identified. Previously some substrates were identified such as WRKY1, ACS2/6, EIN3, WRKY8 and WRKY33 [4, 16, 17]. Here, we showed that MYB13 was identified as a new substrate of MPK3 and MPK6. Functional analyses of plant MYBs indicate that they regulate numerous processes including responses to environmental stress. For instance, MYC2 and MYB2 proteins play important roles as transcription factors in ABA-dependent gene expression under drought and salt stress [18]. The MYB61 are not

induced by ABA, but can enhance drought, salt, or freezing tolerances [19]. Moreover, MYB102 is a key component to integrate signaling pathways in responses of *Arabidopsis* to wounding, osmotic stress [2]. MYB41 controls the short-term transcriptional responses to osmotic stress [20]. MYB44 was published as subtrate of MPK6 and function in seed germination [21]. AtMYB13 has fuction on on the architecture of the inflorescence. The expression of the MYB 13 gene is regulated by dehydration, exogenous abscisic acid, light and wounding [22]. However, the mechanism of biological of MYB13 was not reported. In our data, the interaction of MYB13 and MPK6 was confirmed by pull-down assay. This is the first evidence showed the relationship between MYB13 and environmental stress cascade. Kinase assay confirmed the phosphorylation of MYB13 by MPK3 and MPK6. And the phosphorylation sites were identified at Thr^{41} and Ser^{138}. This is match with well known that MPKs typically phosphorylate their substrate on either a serine or a threonine residue followed by a proline residue (SP or TP). Our data here showed more information and understand of new MPKs substrate in *Arabidopsis*.

5. Conclusion

Mitogen-activated protein kinase (MPK) cascades are signal transduction pathways and play a central role for converting extracellular signals, including environmental stresses, into internal signal transduction and activation of intracellular responses. However, so far only a limited number of target molecules have been identified. Here, we raised a new sign of MYB13, functioned as a new target substrate of MPKs in *Arabidopsis*. MYB13 interacts with MPK3, 4, 6 *in vitro*. MYB13 was phosphorylated by recombinant MPK3 and MPK6. The phosphorylation sites of MYB13 were detected at Thr^{71} and Ser^{138} residues.

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Nghiên cứu nhân tố phiên mã R2R3- MYB (AtMYB13) là cơ chất mới của enzyme kinase MPK3 và MPK6 ở *Arabidopsis*

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Tóm tắt: Mitogen-activated protein kinase (MPK) là con đường truyền tín hiệu phổ biến và rộng rãi trong các sinh vật nhân chuẩn, bao gồm nấm men, động vật và thực vật. Các MPK đóng vai trò trung tâm để chuyển đổi tín hiệu từ ngoại bào, bao gồm áp lực môi trường, thành tín hiệu nội bào và kích hoạt các phản ứng trong tế bào. Đã có nhiều công bố về MPKs ở thực vật được kích hoạt bởi các yếu tố bất lợi từ môi trường như: mặn, lạnh, tổn thương, nhiệt, sốc thẩm thấu, kim loại nặng, tia cực tím, hạn hán và cả các nhân tố gây bệnh sinh học. Tuy nhiên, cho đến nay chỉ có một số ít các cơ chất của nhóm protein kinase này được xác định. Trong nghiên cứu này, chúng tôi đã xác định được nhân tố phiên mã MYB, MYB13 là cơ chất trực tiếp của các MPK trong cây *Arabidopsis*. Sử dụng kỹ thuật pull-down cho thấy protein MYB13 liên kết đặc hiệu với các MPK trong điều kiện *invitro*. MYB13 được phosphoryl hóa bởi protein tái tổ hợp MPK3 và MPK6. Bằng cách đột biến điểm, chúng tôi đã xác định được gốc Thr 71 và Ser138 của MYB13 là vị trí phosphoryl hóa của các MPK. Những kết quả này chỉ ra rằng các protein MPK trực tiếp phosphoryl hóa protein MYB13 trong *Arabidopsis*.

Từ khóa: Nhân tố phiên mã MYB, MAPK, phosphoryl hóa.