Amplification and cloning of arabidopsis 6xhis-tagged mpk6 fusion encoded gene to characterize biochemical mitogen-activated protein kinase in disease resistance role against Fusarium graminearum

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Abstract. The Mitogen-Activated Protein Kinase (MPK) cascade plays an important role in the intracellular signaling transduction pathway leading to resistance against phytopathogens produced by Fusarium graminearum. In the cascade, there are three prominent kinase protein groups involved, an MPK kinase kinase (MPKKK), MPK kinase (MPKK), and an MPK. Recognitions of pathogen-derived molecules in plants trigger rapid activation of some MPKs including MPK6 which are found in a wide variety of plant species, including in Arabidopsis thaliana. The structure of MPK6 contains kinase domain and common docking (CD) domain. CD domain is phosphorylated by interact with MPKK. Moreover, the MPKK which binds to MPK6 and its phosphorylation mechanism are still unknown, so as initial study is needed to investigate biochemical characterization by prepare MPK6 protein. In this research, mpk 6 was amplified by using a pair primer and subsequently was ligated into pET160/GW/D-TOPO vector which contained with sequence encoded 6xHistidine tag protein for protein purification assay.

1. Introduction

Fusarium head blight (FHB) is a destructive disease of cereal crops that have been playing as a major factor in losing both production and quality of cereals in many parts of the world. FHB has the potential to devastate grain crops with the highest negative impact on agricultural economics [1]. It mainly caused by Fusarium species such as Fusarium graminearum. One common feature of many Fusarium species is their ability to produce trichothecenes, a kind of mycotoxins- that is harmful to human health and animals when consuming the contaminated products [2].

Trichothecenes are phytotoxic, and at very lacking level invoke several symptoms such as wilting, chlorosis, and necrosis a generously species of plants [3]. Trichothecenes are separated into four groups (type A, B, C, and D) by their chemical structures with varying degrees of cytotoxic potency [4]. HT-2 and T-2 are the main Type A trichothecenes produced by Fusarium sporotrichioides exhibiting very high toxicity [5]. Deoxynivalenol (DON) as type B
trichothecenes is the most ubiquitous toxin associated with FHB and mainly produced by *F. graminearum* [6]. Meanwhile, type C and D trichothecenes are infrequent and are not affect FHB disease [7].

Trichothecenes interrupt protein synthesis by interacting with the peptidyltransferase site of the 60S ribosome subunit in plant and, consequently, block translational initiation or elongation [8]. Inhibitors of the peptidyltransferase reaction can drive a ribotoxic stress response that activates the roles in the defense signaling cascade [9], mitogen-activated protein kinase (MAPK) components that regulate cell survival in response to stress [10]. The actuating of MPKs by ribotoxins triggers both inflammatory gene expression and macrophage apoptosis, making the innate immune system a critical target for these factors [9]. The preferred plant system performing so far a larger picture of the various functions of MAPKs in the model plant species *Arabidopsis thaliana* [11]. The Arabidopsis genome sequence information allowed a type of MAPK-related kinases based on sequence homology with other plants including crop plants [12].

Kinase signalings of the mitogen-activated protein kinase (MAPK) type play a remarkably important role in plant signaling. MAPK pathways in a plant are three-tiered signaling kinase components that function downstream of receptors and relay extracellular stimuli onward into intracellular reactions while at the same time multiplying the transducing signal [13]. The certain signal is achieved by a MAPK cascade of three main transduction arranged, interacting types of kinase proteins. MPK activity is evoked upon phosphorylation by MPK kinases, which are in turn phosphorylation activated by MPKK kinases [14]. The Arabidopsis genome contains 60 MKKKs, 10 MKKs, and 23 MPKs (according to systemic nomenclature, the MAPKKKs, MAPKKs, and MAPKs in Arabidopsis were called MKKKs, MKKs, and MPKs). Interestingly, among these protein kinases, MPK6 is related to disease resistance against *Fusarium spp*. and it is orthologs in other plant species [15]. The insights into MAPK-mediated pathogen defense response regulation with a particular focus on the cascades involving MPK6 which acts as positive mediators of defense responses in Arabidopsis species [11].

Activated MPK6 phosphorylates various of downstream substrates, including transcription factors and cellular structural proteins. It is phosphorylated by upstream MKKs [16]. The complexity of the upstream MKKs and downstream substrates emphasized that plant MPKs have an exclusive mechanisms to accurately interact with these MKKs and substrates [17]. MPK6 is phosphorylated by upstream MKKs, and in turn, it could phosphorylate its substrate. However, the specific upstream MKKs that possess regulation in this MPK6 phosphorylation remains mostly elusive and very limited known.

The MAPK structures consist of substrate binding sites in the C-terminal domain [18]. Activation MPK6 is determined by the specificity of interactions between its D-motif binding site and the docking motifs in substrates, MAPKKs, and phosphatases. The D-motif binding site consists of the common docking domain (also known as the CD-domain) and hydrophobic docking groove. CD domain is phosphorylated by interact with MPKK [19]. Moreover, the MKK which binds to MPK6 and its phosphorylation mechanism are still unknown, thus, as an initial study is needed to investigate biochemical characterization by prepare MPK6 protein.

### 2. Materials and Methods

#### 2.1 Amplification of *mpk6*

The isolated *Arabidopsis thaliana* DNA were used as a template for amplifying coding sequence of *mpk6* (1188 bp) by PCR method. Primer was designed based on the sequence from NCBI (National Centre of biotechnology Information) database (Accession number : NC_003071). The amplicon was subsequently synthesized by using the following primer: *mpk6 forward* (5' - GACGGTGGTTCACGTTC - 3’) and *mpk6 reverse* (5’ – TTGCTGA TATTCTGGATTGA - 3’).

The PCR reaction included 25 cycles with denaturation temperature: 94°C, annealing temperature:
55°C and elongation temperature: 68°C. The amplicons were resolved by electrophoresis on 1% agarose gel in Tris-acetat acid-EDTA (TAE) 1X buffer along with 1 kb DNA marker.

2.2. Cloning into pBlueScript vector

The PCR fragments were purified and ligated into pBlueScript vector. The cloned plasmid (pBlueScript–mpk6) was chemically transformed into Escherichia coli DH10B for plasmid maintenance. Transformation was based on blue-white screening as a result of growth of transformant in the medium with ampicillin and the reaction of galactoside (expressed by lacZ gene of pBlueScript) in X-gal medium. A positive clone was indicated by white colonies that can grow in medium with ampicillin, since the successfull of ligation and transformation was indicated by interuption in lacZ gene and resistancy to ampicillin. Plasmids were isolated from white colonies and then were purified, analysed with restriction digest and sequenced by Automated DNA Sequencer.

2.3. Cloning into pET160/GW/D-TOPO vector

MPK6 truncations were PCR-amplified from the previously constructed pBlueScript-mpk6 vector. The PCR fragments were cloned into the pET160/GW/D-TOPO vector, then A 6×His tag was fused to the N-terminus of MPK6. The resulting constructs were all confirmed by sequencing and transformed into E. coli BL21 strain.

3. Result and Discussion

Initially, in this study, we amplified the Arabidopsis mpk6 Gene by PCR method and the resultant band of expected sizes was detected by the quite clearly shown as the figure 1. Therefore, the PCR product cloned with the pBlueScript vector in the E.coli DH10B strain. The sequence of PCR products was confirmed by sequencing. Sequencing analysis was done by using BLAST (Basic Local Alignment Search Tool) in NCBI showed that the length of the gene was approximately 1188 bp.

![Figure 1](image)

**Figure 1.** PCR product of the Arabidopsis mpk6

As shown in figure 1 PCR amplification encoded gene of mpk6 was successfully isolated and ligated into pBlueScript vector. It was represented by colony PCR to confirm the mpk6 gene insertion (figure 2). The pBlueScript vector using for generating many copies of mpk6 gene that can use as PCR template for the future study in this research.
Figure 2. Confirmed the mpk6 gene insertion in the pBlueScript vector by PCR from the positive clone E.coli DH10B strain

Mpk6 truncations were PCR-amplified from the previously combination pBlueScript -mpk6 vector. The PCR fragments were cloned into the pET160/GW/d-TOPO vector (Invitrogen) and then transformed into E. coli BL21 strain. pET160/GW/d-TOPO vector as a donor vector in these Gateway system contained unique site and purification tag polyhistidine (6xhis) region. the sites vector has a specific site of recombination (“att” sites) which are much longer (25–242 bp) than restriction sites in regular vector with restriction enzyme cloning [20]. The mpk6 encoded gene was successfully ligated between the att site. Moreover, there were twice as many reports argue what is better in the existence of spacers encoded in att sites (attB) or those encoded in multiple restriction cloning sites. In our hand, the sink or swim of a functional technique involving translational fusion does not rely on a particular cloning strategy, but instead kind of a case-by-case basis. It owing to we examined cloning mpk6 truncated with pBlueScript vector as well in this research.

Figure 3. Confirmed the 6xhis tag-mpk6 fusion gene insertion in the pET160/GW/d-TOPO vector by colonyPCR

Furthermore, the polyhistidine (6xhis) tag which bound to the mpk6 is a key of the assay in process of recombinant protein expression or purification in order to characterize the biochemical structure of mpk6 in the further study. The mpk6 fragment which was cloned into the pET160/GW/D-TOPO vector, then A 6xHis tag was fused to the N-terminus of mpk6, subsequently confirmed by colony PCR of 12 positive colonies (figure 3). And the result delineates mpk6 successfully fused to 6xHis tag in pET160/GW/D-TOPO vector.
4. Conclusions
The Arabidopsis 6XHis-tagged MPK6 Fusion Encoded Gene has been successfully amplified and cloned into pET160/GW/D-TOPO vector which will use for to characterize Biochemical Mitogen-Activated Protein Kinase in Disease Resistance Role against Fusarium graminearum for the further research.

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