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CHARACTERIZATION OF RICE MAP KINASE SUBSTRATES

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Abstract

The mitogen-activated protein (MAP) kinase cascade plays an important role in the regulation of plant growth and development as well as biotic and abiotic stress responses. Rice MAP kinase 5 (OsMPK5), an orthologue of *Arabidopsis* AtMPK3, is an abscisic acid- and stress-inducible kinase that positively regulates rice tolerance to abiotic stresses but negatively modulates host resistance to pathogen infection. However, it is unknown how OsMPK5 regulates downstream signaling components and influence rice biotic and abiotic stress responses. To elucidate the mechanism of OsMPK5-mediated signal transduction in rice, therefore, it is critical to identify its potential protein targets or substrates. In this study, an in-situ solid phase phosphorylation screen was conducted to isolate potential OsMPK5 substrates. A rice protein expression library was screened using purified recombinant OsMPK5 and ^{32}P γ-ATP. The phosphorylation screen yielded over a dozen putative substrates with potential roles in apoptosis, ion transportation, transcription, phosphorylation and dephosphorylation, etc. Strikingly, many of these putative substrates or their orthologues from other plant species have been previously shown to be involved in biotic and abiotic stress responses. In this study, two of the putative substrates were further characterized by a combination of molecular, biochemical and genetic approaches.

The first putative substrate is a transcription factor called OsEIL1, which is an important signaling component of ethylene signal transduction. In vitro protein binding and kinase assays reveal that OsMPK5 interacts with and phosphorylates OsEIL1 in vitro. In addition, OsMPK5 protein kinase activity is negatively correlated with the OsEIL1

protein level and downstream *OsERF1* and *OsPR5* expression in transgenic rice. In addition, transgenic analysis showed that OsEIL1 positively regulated OsERF1 and OsPR5 expression and increased rice disease resistance against the rice blast infection. This suggests that OsMPK5 mediates biotic stress responses by negatively regulating OsEIL1 stability and ethylene signaling transduction.

The second interacting protein is a rice calcium-dependent protein kinase (OsCDPK5) which belongs to a large family of protein kinases with diverse roles in plant growth and development as well as biotic and abiotic stress responses. *In vitro* kinase assays demonstrated the phosphorylation of OsMPK5 by OsCDPK5 in a calcium-dependent manner. The autophosphorylation activity of OsCDPK5 as well as its ability to phosphorylate OsMPK5 were positively regulated by calcium concentration in vitro. Furthermore, OsCDPK5 was found to physically interact with OsMPK5 *in vitro* and *in vivo*. RT-PCR and RNA blot analyses showed that the expression of *OsCDPK5* in rice was up-regulated by ACC and wounding but down-regulated by ABA. To determine the potential role of *OsCDPK5* in plant stress tolerance, transgenic RNAi lines were generated via the *Agrobacterium*-mediated rice transformation. Similar to the silencing effect of *OsMPK5* in rice, suppression of *OsCDPK5* enhanced the expression of ethylene-responsive defense genes, suggesting that OsCDPK5 may potentially regulate rice biotic and abiotic stress tolerance.

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Chapter 1 Literature Review

Rice has been cultivated for thousands of years and is currently the main staple

food for about a half of the world's population. Besides its economic importance, rice is

also an excellent model for monocots, particularly cereal crops, due to its completely

sequenced genome, well-established techniques for gene transformation, and various

genetic resources and tools for forward and reverse genetic analyses (Jung et al., 2007).

With the explosive increase of the world population and deteriorating environments (e.g.,

scarce fresh water, extreme temperatures), there is an urgent need to significantly

increase rice production. As a result, it is important to understand rice defense

mechanisms and to make rice cultivars more tolerant to biotic and abiotic stresses.

1.1 Plant biotic and abiotic stress responses

Plants are constantly exposed to various biotic (e.g., pathogen and insects) and

abiotic (e.g., drought and salinity) stresses. In the plant-pathogen interaction, host defense

response is often manifested as hypersensitive response (HR) and systemic acquired

resistance (SAR). HR is characterized by the localized host cell death at the site of

infection whereas SAR is a long lasting systemic resistance throughout the entire plants.

Upon microbial infection, the basal defense is often triggered by pathogen-associated

molecular patterns (PAMPs). During the coevolutionary process of host-pathogen

interaction, the pathogen acquired the ability to deliver its effectors into plant cells to

suppress PAMP-triggered innate immunity and facilitate pathogen growth and disease

development. On the other hand, host plants developed surveillance proteins (e.g., NBS-

LRR proteins encoded by many disease resistance genes) to detect and block the pathogen effectors, which often leads to effector-triggered immunity or race-specific disease resistance (Nürnberger and Brunner, 2002). Both PAMP-triggered and effector-triggered immunities have been shown to be associated with the mitogen-activated protein kinase (MAPK) cascade. The most complete understanding of the PAMP-triggered immunity involves the perception of bacterial flagellin (a peptide elicitor) by the plant leucine-rich repeat (LRR) receptor kinase, activation of a MAPK cascade, and induction of WRKY transcription factors and downstream defense genes (Asai, 2002; Fobert and Després, 2005).

Abiotic stresses, such as drought, salinity, or low and high temperature, usually cause plant metabolic toxicity, membrane disorganization, generation of reactive oxygen species (ROS), inhibition of photosynthesis and altered nutrient acquisition (Hasegawa et al., 2000; Sreenivasulu et al., 2007). In response to these adverse environmental stresses, plants need to adapt constantly by modifying stress signaling pathways and thus regulating stress-responsive gene expressions. For example, protein kinases are often activated by external stresses, resulting in changes in protein phosphorylation, transcription and physiological adaptation. In Arabidopsis, AtMKK1 (a MAP kinase kinase) can be activated by wounding, cold, drought and salinity, and subsequently enhances AtMPK4 (a MAP kinase) activity by phosphorylation. Suppression of AtMPK4 expression decreased *Arabidopsis'* tolerance to abiotic stresses. Inversely, AtMPK4 is also involved in a negative regulation of disease resistance (Desikan et al., 2001). In addition, data from transcriptome analyses indicates that abiotic stresses induce numerous

genes that encode proteins ranging from membrane-associated transporters, key enzymes for osmolyte biosynthesis, to transcription factors and protein kinases (Lilly et al., 2002).

Increasing evidences suggest that there are extensive cross-talks between biotic and abiotic signaling pathways (Fujita et al., 2006). High humidity and high temperature were shown to promote pathogen growth while attenuating the plant disease resistance. For example, Arabidopsis ssi4 (encoding a NBS-LRR protein) mutant display lesion-mimic and disease resistance under the normal growth condition (Zhou et al., 2004). However, high humidity inactivates AtMPK3/6 kinase activities and suppresses the cell death and disease resistance function. condition, however, both cell death and disease resistance are inhibited. In rice plants, an abscisic acid-inducible MAP kinase (OsMPK5) was found to positively regulate abiotic stress tolerance, but negatively modulate disease resistance against pathogen infection (Xiong and Yang, 2003).

1.2 Role of plant hormones in mediating biotic and abiotic cross-talk

A large body of evidence demonstrates that plant hormones such as ethylene (ET), abscisic acid (ABA), jasmonic acid (JA) and salicylic acid (SA) play important roles in biotic and abiotic stress responses (Alonso and Ecker, 2001; O'Donnell et al., 2003)**.** The biosynthesis and signal transduction of these hormones are often activated in response to a variety of stresses and their interplay is important for the coordinated expression of stress responsive genes and appropriate physiological adaptation. At the level of hormone biosynthesis, ABA and ET were shown to be mutually inhibitive (Gazzarrini and McCourt, 2001). ABA may also block the SA-dependent defense response because ABA-deficient tomato mutant *sitiens* exhibited the SA-mediated responses (Asselbergh et al.,

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2007). The exogenous application of ABA also down-regulates JA and ET-responsive gene expression, whereas the expression of these defense genes was increased in ABA-deficient mutants (Anderson et al, 2004). Furthermore, arabidopsis era3 mutant (with enhanced response to ABA) is allelic to ET insensitive 2 (EIN2) mutant, suggesting possible crosstalk between ethylene and ABA signaling pathways (Ghassemian et al., 2000). In general, SA, JA and ET play positive roles against pathogen infection (Broekaert et al, 2006). By contrast, ABA often plays a negative role in disease resistance, but is important for positive regulation of plant response to abiotic stresses such as drought, salinity and cold (Jwa et al., 2006). However, the molecular mechanism mediating the cross-talk between the ET and ABA pathways remains to be elucidated.

1.3 Ethylene biosynthetic and signaling pathways

Ethylene regulates numerous physiological processes, including seed germination, root-hair initiation, leaf and flower senescence and abscission, fruit ripening, and plant responses to a wide variety of stresses (Bleecker and Kende, 2000). The biosynthesis of ET involves two main steps: the conversion of S-adenosyl-L-Met to 1-aminocyclo-propane-1-carboxylic acid (ACC) and the oxidative cleavage of ACC to form ethylene (Kende, 1993; Zarembinski and Theologis, 1994). ACC synthase (ACS) and ACC oxidase (ACO) respectively catalyze the above two steps. Usually, the basal level activity of ACS is very low in tissues that produce a low amount of ethylene. Hormone treatments, such as auxin, enhance ACS activity, causing a significant increase of ET production (Yang and Hoffman, 1984). MAPKs activities are required to keep a constitutive ET

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biosynthesis. AtMPK3/6 plays a positive role in ethylene production by phosphorylating

ACS2/6 and increasing the stability of both enzymes (Liu and Zhang, 2004).

In Arabidopsis, ET signal transduction is composed of several signaling

components that involve the formation of the triple-response phenotype (a short,

thickened root and hypocotyl with exaggerated curvature of the apical hook). These

components include receptors (ETR1, ETR2, EIN4, ERS1, ERS2), constitutive triple

response 1 (CTR1), and ethylene insensitive genes (EIN2 and EIN3) (Benavente and

Alonso, 2006). CTR1 is a negative ethylene response factor, a member of MAPKKK

which in the absence of ET, represses ET-inducible phenotypes, such as the triple-

response phenotype (Kieber et al., 1993). EIN2 is a membrane-associated protein which

plays a pivotal role by positively regulating ET signal transduction (Wang et al., 2007).

EIN3 is an early ET-responsive transcription factor located downstream of EIN2

(Stepanova and Alonso, 2005). ERF1 has been demonstrated as a direct target of EIN3

(Solano et al., 1998). In Arabidopsis, the ein3 mutant shows a decreased expression of

ERF1 and EBF1/2 (EIN3 binding factor) that can be regulated by various stress signals,

such as ethylene and ABA (Solano et al., 1998; Wang et al., 2007).

EIN3 and its homologs, EIN3-like transcript factors (EILs), are regulated at the

post-translational level in response to exogenous ET treatment. The Arabidopsis ein2

mutant exhibits an increased level of EIN3 protein but no change in its mRNA expression

(Guo and Ecker, 2003). EIN2 is not known to physically interact with EIN3. At present,

it is unclear how EIN2 may regulate EIN3 protein level. Since CTR1 has a similar

function and structure to MAPKKK, MAPK cascade may directly or indirectly act on the

ethylene signaling pathway (Hamel et al., 2006). Most recently, AtMPKK7/9-AtMPK3/6

cascades was shown to modify ethylene signaling pathways by phosphorylating EIN3 protein (Yoo et al., 2008). Furthermore, EBF1/2 (F-box subunit of the SCF complex) was involved in the feedback regulation by recruiting EIN3 for degradation (Potuschak et al., 2003). The single or double mutant of EBF1/2 was found to have a higher EIN3 protein level.

Ethylene responsive element binding factors (EREBPs) are regulated by various stresses and hormones besides ethylene. EREBPs are the secondary transcription factors binding the cis-element GCC box (Solano et al., 1998). ERFs (ethylene responsive factors) are members of the EREBP family and play diverse roles in plant adaptation to environmental stresses, such as drought, cold, salt, oxidative damage, and ABA treatment (Tang et al., 2006). For example, the barley ERF protein HvRAF enhances salt tolerance in Arabidopsis (Jung et al., 2006). In tomatos, the mRNA level of jasmonate and ethylene response factor1 (JERF1) is regulated by ET, MeJA, ABA, and NaCl treatment. JERF1 modulates the expression of GCC box-containing and salt tolerance genes (Zhang et al., 2004). Some ERF proteins may act as negative regulators in plant stress responses. Arabidopsis ERF4 and ERF7 act as transcriptional repressors to modulate ethylene and ABA responses (Song et al., 2005; Yang et al., 2005). The expression of GbERF2 in *G. barbadense* was reduced by ABA treatment, which leads to the accumulation of pathogenesis-related (*PR*) gene transcripts, such as *PR2* and *PR4* (Zuo et al., 2006).

1.4 Abscisic acid biosynthesis and signaling pathways

ABA inhibits seed germination, root growth, stomatal opening, and play a central role in abiotic stress responses (Koornneef et al., 1984; Leung et al., 1997; Allen et al.,

1999; Hoth et al., 2002). The endogenous ABA level in plants is regulated by ABA biogenesis and degradation pathways. In its biosynthetic process, three major genes have been identified, which encode zeaxanthin epoxydase, 9-cis epoxycarotenoid dioxygenase, and abscisic aldehyde oxidase (Giraudat et al, 1994). In the degradation process, P450 CYP707 family enzymes in Arabidopsis can hydroxylate ABA at C-8' into phaseic acid, which is the inactive form of ABA. In addition, ABA glycosyltransferase may catalyze the conjugation of ABA into the inactive ABA glucosyl ester. This process can be reversed by AtBG1 (Arabidopsis β-glucosidase) which releases ABA from this inactive glucosyl ester (Finkelstein and Gibson, 2002; Hirayama and Shinozaki, 2007).

Recently, three ABA receptors have been identified (Wang and Zhang, 2008). The first receptor, ABA-binding protein (ABAP1), was identified in barley. The flowering-time control protein (FCA) is the closest Arabidopsis homolog of ABAP1. FCA is a nuclear receptor that binds to FY, an RNA processing factor, to inhibit FCA and Flowering Locus C (FLC) expression (Razem et al., 2006). The Mg-chelatase H subunit (ABAR) is a plastic receptor controlling major ABA signaling and involved in chlorophyll synthesis and plastid-to-nuclear retrograde signaling (Sheng et al., 2006). The last one is G protein coupled receptor2 (GCR2), a plasma membrane receptor predicted to have seven membrane-spanning domains, which, like other G protein-coupled receptors, interacts with a subunit (GPA1 in Arabidopsis) of trimeric G protein (Liu et al., 2007; Verslues and Zhu, 2007; Hirayama and Shinozaki, 2007).

Several ABA insensitive genes were identified by screening the mutant seeds capable of germination and good seedling growth in the presence of ABA concentrations that are inhibitory to the wild type. These genes include *ABI 1/2/3/4/5*. Both *ABI1* and

ABI2 genes encode serine/threonine protein phosphatase 2C (PP2C) (Schweighofer et al., 2004). The abi1 or abi2 mutant is dominant and leads to ABA-insensitive seed germination, indicating that ABI1/2 are the negative regulators of ABA signaling in seed germination. ABI3/4 are two transcription factors activating or repressing ABA dependent and independent gene expressions in seeds (Holdsworth et al., 2001; Zeng et al., 2003). ABI5 is another transcription factor coding for a basic leucine zipper (bZIP) that binds to the ABA responsive cis-acting element (ABRE) of ABA-induced gene promoters (Finkelstein and Lynch, 2000). The physical interaction of ABI3 and ABI5 has been demonstrated and appears to be important for the transcriptional activation of downstream genes. The germination of the abi4 mutant is insensitive to ABA and sugar (Teng, 2008). Interestingly, ABI4 contains the greatest sequence homology with ethylene-responsive element binding proteins (EREBP-type transcription factors), and contains an APETALA2 (AP2)-like DNA binding domain characteristic of AP2/EREBP family transcriptional regulators (Finkelstein et al., 1998; Ohta et al., 2000; Soderman et al., 2000). Furthermore, ETR1, CTR1 and EIN2 negatively regulate ABA signaling, indicating that ET and ABA signaling pathways may share common components (Ghassemian et al., 2000).

The signaling networks among various plant hormones can be elucidated by the combination of forward and reverse genetic strategies. Decades of genetic and physiological studies have identified most receptors of plant hormones and the components of hormone signaling pathways. To understand biotic and abiotic crosstalks, however, we need to further elucidate the molecular interactions among these hormonal pathways. Undoubtedly, many factors could function as link points among different

signaling pathways. From the above review, we know that MAPK cascades are involved in plant defenses and hormone perception pathways. MAPK cascades may function as the molecular switches by converging signaling from various stresses and relaying to their downstream targets (Takken et al., 2006).

1.5 Mitogen-activated protein kinase cascades

Mitogen-activated protein kinase (MAPK) cascades are vital to fundamental physiological functions (Sheng, 2003). Each species of yeasts, animals or plants may have multiple MAPK cascades, functioning in different ways to regulate hormone responses, cell cycle regulation, abiotic stress signaling, and host defense mechanisms. MAP kinases are activated directly by their up-stream kinases. MAPK cascades are a group of interacting protein kinases consisting of at least three components including MAPKKKs, MAPKKs, and MAPKs. They play roles as convergent points by receiving up-stream signals and regulating eukaryote activity by modifying downstream factors (Zhang and Klessig, 2001). The cascade undergoes a sequential phosphorylation from MAPKKK to MAPK, which means MAPKK, a phosphorylational substrate of MAPKKK, phosphorylates MAPK and activates its kinase activity.

Besides the positive regulation of MAPK activity, MAP kinase activity can also be negatively mediated by protein kinase and phosphatase that are transcriptionally increased by MAPKs (negative feedback regulation). In the ET signaling pathway, CTR1, a homolog of MAPKKK, inhibits AtMPK3/6 kinase activity through AtMPKK7/9 (Yoo et al., 2008). Inactivation of MAP kinases by phosphatase involves dephosphorylation of threonine and tyrosine residues on the T-x-Y motif within the activation loop (Sheng,

2002). In plants, several protein phosphatases and their homologs have been characterized. These enzymes include members of the PP2C (protein phosphatases 2C), PTP (Protein Tyrosine Phosphates), and DSP (Dual-Specificity Phosphatases) families. DSPs are members of a subfamily of PTPs that contain a short conserved signature motif around their catalytic sites (Kerk, 2002; Tena et al., 2001). A total of 11 DSPs have been found in the *Arabidopsis* genome. The *Arabidopsis* protein phosphatase ABI1 (ABA insensitive 1), which belongs to PP2C, plays an important role in the ABA signaling pathway and is thought to dephosphorylate a component of the MAP kinase cascade. Another phosphotase AtMKP1 (*Arabidopsis* MAP kinase phosphatase, member of PTP family) preferentially interacts with AtMPK6 and, to a lesser extent, with AtMPK3 and AtMPK4 (Mishra et al., 2006).

1.6 The evolution of MAPKs

MAPK cascades have adapted their specific structures and functions in different species under evolutionary selections. MAPK cascades are conserved in their functional structures from MAPKKKs to MAPKKs and to MAPKs, based on the studies ranging from single cell eukaryotes (S.cerevisiae) to mammalian animals. All MAPKs contain triplet T-x-Y which is targeted and phosphorylated by MAPKKs. However, all were shaped by evolutionary pressure in multicellular eukaryotes, and thus contain certain functions only in animals or plants, no matter whether these MAPKs underwent evolutionary radiation starting before multicellular eukaryotes emerged, or are only found in multicellular eukaryotes (Kultz, 2001). For example, osmotic selection is the most ancient stress shaping MAPK evolution. MAPKs found in protozoans are ancient

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MAPKs with TEY or TDY triplets, similar to the eukaryote MAPK3. MAPK3

homologies in eukaryotes are called ERKs and SAPKs. In yeast, the only member of

SAPK Hog1 (High osmolarity glycerol 1) is a TGY protein kinase, which is necessary for

cell survival in hyperosmotic conditions (Chen and Thorner, 2007). But, in animals as in

the nematode *Caenorhabditis elegans* and the fly *Drosophila melanogaster*are, at least

two *SAPK1* (TPY) and two *SAPK2* (TGY) genes were identified thus far. Mammals have

at least seven *SAPK* genes, three for *SAPK1* and four for *SAPK2*. Plant ERKs have

evolved the capacity to function in osmosensory signal transduction pathways that are not

controlled by ERK but by the SAPK subgroup in animals and yeast, thereby

compensating for the lack of plant SAPKs (Kultz and Burg, 1998). Evolution shaped the

number and structure of MAPKs that display specific functions in plants. Elucidating

their specific roles may provide valuable clues for understanding their evolutionary

processes.

1.7 Plant MAPKs involved in biotic and abiotic stress responses

Most current research on plant MAPK focuses on elucidating MAPKs' responses

to stresses and hormones and their roles in regulation of plant hormone and stress

signaling pathways. In plants, MAPKs belong to a large family of protein kinases with

essential functions for plant development and stress responses. Similar to yeast and

animals, their kinase activities are activated by MAPKKs through the phosphorylation of

their triplets (T-X-Y). However, plants have to endure environmental challenges because

they are sessile and have evolved various regulatory systems in response to biotic stresses

and abiotic stresses.

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Biotic stresses frequently activate the MAPK cascades. For example, plant resistance response involves not only the production of ROS, but also the activation of MAP kinase pathways (Zhu, 2001). Upon infection by *Pseudomonas syringae*, tomato MAPKKs (including MEK1 and MEK2) and MAPKs (including NTF6 and WIPK) are activated in response to the AvrPtoB effectors secreted from *Pseudomonas syringae*. The activated MEK1/2-NTF6/WIPK cascade enhances pathogen resistance by regulating the expression of SAR components such as NPR1 and two transcription factors (TGA1a and TGA2.2) (Zhu, 2001; Ekengren, 2003). Upon infection with tobacco mosaic virus, tobacco MAPKs (such as SIPK/Ntf4/WIPK) pathways are activated, which results in hypersensitive cell death as well as loss of membrane potential, electrolyte leakage and rapid dehydration (Liu et al., 2007).

Abiotic stresses also activate the MAPK cascades. In an Arabidopsis MAPK cascade including AtMKK1/2 and AtMPK4, protein levels and kinase activities are up-regulated in response to cold, salt, H_2O_2, JA and ethylene. This cascade functions as a negative regulator of systemic acquired resistance and a positive regulator for salicylic acid-mediated tolerance (Petersen et al., 2000; Broderson et al., 2006; Schweighofer et al., 2007; Huang et al., 2000; Teige et al., 2004). Another typical example in Arabidopsis is the MEKK1-MEK4/5-MPK3/6 cascade. MEKK1, one of the MAPKKK, can phosphorylate MEK4 and its redundant protein MEK5. Both MEK4 and MEK5 can phosphorylate MPK3 and MPK6. This cascade receives a signal from FLS2 (flagellin receptor) upon interacting with flagelin (a bacterial elicitor) (He et al., 2006; Zhang and Klessig., 2001). In addition, AtMPK3 plays a role in abscisic acid-induced post germination growth arrest (Lu et al, 2002). The silence of AtMPK6 compromises basal

resistance (Menke et al., 2004). Both AtMPK3 and AtMPK6 can prompt ethylene synthesis by phosphorylating ACS2/6 (members of ACC synthesase family) and promoting both enzyme protein stabilities (Liu and Zhang, 2004).

In cases other than Arabidopsis, several MAPKs from different species have been found to respond to hyperosmotic or hypoosmotic stress. In alfalfa cells, a 46-kDa MAPK, named salt-stress-inducible MAP kinase (SIMK), becomes activated upon hyperosmotic stress treatments. Its hyperosmotic induced activity also is enhanced by SIMKK through phosphorylation (Kiegerl et al., 2000). In tobacco, SA-inducible protein kinase (SIPK, an orthologue of AtMPK6) is activated by both hyperosmotic and hypoosmotic stresses, indicating that an additional modification of SIPK may exist. Using immunoprecipitation followed by two-dimensional in-gel kinase assay, three forms of SIPK was observed. This indicates that a specific modification of SIPK occurs in response to the specific stress (Droillard et al., 2000).

1.8 MAPKs in rice

A total of 17 MAP kinase genes have been identified from the rice genome (Reyna and Yang, 2006). Their predicted protein products all share eleven conserved amino acid domains (I-XI) with the molecular masses in the range of 42-79 kDa (Xiong and Yang, 2003). All of these rice MAP kinases contain the triplet Thr-X-Tyr (except OsMAPK2, where it is Met-X-Tyr) with either TEY or TDY motif as a phosphorylation site by MAPKKs. These 17 MAPKs can be divided into six subgroups (A, B, C, D, E, and F). However, the specific function of each member or each group in biotic and abiotic tolerance remains to be elucidated (Rohila and Yang, 2007).

1.9 Role of OsMPK5 and its orthologues from other plants

OsMPK5 (a rice orthologue of AtMPK3 and NtWIPK) is inducible at the mRNA, protein and kinase activity levels by ABA as well as biotic (fungal and bacterial infections) and abiotic (wounding, drought, salt and cold) stresses (Xiong and Yang, 2003). Transgenic analysis demonstrated that *OsMPK5* RNAi line plants showed a significant reduction in drought, salt and cold tolerance, but exhibited a constitutive expression of pathogenesis-related (PR) genes and an enhanced resistance to fungal (*M.grisea*) and bacterial (*Burkholderia glumae*) infection. Overexpression of OsMPK5 led to an enhanced tolerance to abiotic stresses (cold, salt and drought) (Xiong and Yang, 2003). In addition, OsMPK5 appears to up-regulate ABA but down-regulate ethylene production (Yang, not published). In *Arabidopsis*, AtMPK3/6 were shown to regulate ACS2/6 stability and activity by phosphorylation, resulting in an increases of ethylene production (Zhang et al., 2004). These results suggest that OsMPK5 could play different biological roles from AtMPK3/6.

Only two members are included in group A of the rice MAPK family. The second one is OsMPK1, the orthologue of AtMPK6 (Reyna and Yang, 2006). Even though AtMPK3 and AtMPK6 play a redundant role in *Arabidopsis* defense signaling (Miles et al, 2005), it is currently unclear if OsMPK1 and OsMPK5 have redundant function. One interesting finding is that the suppression of *OsMPK1* (*OsMAPK6*) results in the increased expression of *OsMPK5* gene (Lieberherr et al., 2005). This observation suggests potential functional compensation between OsMPK1 and OsMPK5.

The most extensively studied OsMPK5 orthologues are AtMPK3 and AtMPK6 in Arabidopsis. As mentioned above, AtMPK3 plays a redundant role with AtMPK6. Their kinase activities are activated by up-stream AtMKK4/AtMKK5. AtMKK4/5-AtMPK3/6 pathway positively regulates ethylene synthesis through phosphorylating ACS (ACC synthase) (Liu and Zhang, 2004). A recent report indicates that the AtMKK7/9-AtMPK3/6 cascade bifurcates the regulation of the ethylene signaling pathway by phosphorylating EIN3 and regulating ethylene responsive gene expressions (Yoo et al., 2008).

In tobacco, WIPK and SIPK modify transcription factors and expressions of *PR* genes. WIPK is the wounding induced protein kinase, an ortholog of OsMPK5 (Zhang et al., 2001). WIPK and SIPK coorperatively regulate JA synthesis (Seo et al., 2007). Both WIPK and SIPK are activated by NtMEK2 (*Nicotiana tabacum* MAPK kinase 2) and induce hypersensitive cell death as well as expression of *PR* genes such as *PR-1a, PR-1b, PR-2* and *HMGR* (Yang et al. 2001). Furthermore, the activated WIPK can increase expression of WRKY transcription factors (Kim and Zhang, 2004), which can be specifically phosphorylated by SIPK (Menke et al., 2005).

Although OsMPK5 and its orthologues have been well studied during the past decades, relatively little is known about their substrates and downstream target proteins. To elucidate the OsMPK5-mediated signaling pathway and its role in biotic and abiotic stress response, it is essential to identify and characterize the potential substrates of OsMPK5. The major objective of this study is to isolate putative substrates of OsMPK5 using an *in-situ* solid phase phosphorylation screen and to characterize these putative substrates using a combination of molecular, biochemical and transgenic approaches.

Chapter 2 Identification of OsMPK5 Substrates

Using *In-Situ* Solid Phase Phosphorylation Screening

2.1 Abstract

OsMPK5 is an ABA-inducible MAP kinase that positively regulates rice tolerance to abiotic stresses but negatively modulates host resistance to pathogen infection. However, it is unknown how OsMPK5 regulates downstream signaling components to influence rice biotic and abiotic stress responses. To elucidate the mechanism of OsMPK5-mediated signal transduction in rice, it is critical to identify its potential protein targets or substrates. In this study, an *in-situ* solid phase phosphorylation screen was conducted to isolate potential OsMPK5 substrates. A rice protein expression library was screened using purified recombinant OsMPK5 and ^{32}P γ-ATP. The phosphorylation screen yielded a dozen of potential substrates, which can be classified into different functional categories that involve cell apoptosis, ion channel, transcription, phosphorylation and dephosphoruylation, etc. For example, one of the substrates or interacting proteins is the rice calcium-dependent protein kinase 5 (OsCDPK5). Using an *in vitro* kinase assay, we further confirmed the phosphorylation event between OsCDPK5 and OsMPK5, demonstrating the utility of the solid phase phosphoaylation screening for identifying plant MAPK substrates.

2.2 Introduction

Biochemical screens are becoming an important tool to identify numerous substrates of protein kinases, even though genetic screen is probably still the most powerful approach to determine specific gene function and associated phenotype. With the progressive maturation of methods in examining protein-protein interaction, biochemical screens have been very popularly utilized to elucidate interactions among proteins and their biochemical functions in various signaling pathways.

Interaction screen and phosphorylation screen for kinase substrates have been widely performed in yeast and animal systems (Thomas et al, 2000; Casci, 2006). Interactional screen is based on two protein binding activity. Practically, the screen of peptides has been performed to determine the consensus phosphorylation motif of a kinase, and further identify the protein substrates by searching protein peptides data base (Schulze, 2004; Hunter, 2004). In addition, yeast two-hybrid is a useful method to directly identify potential physiological substrates binding to the kinase (Parrish et al, 2006). In certain situations, however, protein kinases may weakly interact with their substrates. Their substrates may not be able to be screened only based on their binding activities. In that case, phosphorylation screen (a powerful functional screen) is a useful supplement for identifying the direct protein kinase substrates without the requirement of the tightly binding activities between protein kinases and their specific substrates (Fukunaga and Hunter, 1997). Phosphorylation screen is designed primarily based on the understanding that the purified kinases can maintain the relatively stable structures and activities.

MAP kinases from yeasts and animals have been successfully used to perform phosphorylation screen. The regulatory mechanisms of MAP kinase activities are conserved among plants and animals even though they display distinct biological functions (Pearson et al, 2001). MAP kinase activities usually are greatly activated by its up-stream signaling or directly by MAPKKs, the immediate up-stream kinase of MAPKs. However, most MAPKs contain their kinase activity *in vitro* that provides the possibility to be used to screen their substrates (Zhang and Klessig, 2001). For example, ERK1 MAP kinase purified from *E.coli* was used to perform an *in-situ* solid phase phosphorylation screen for its substrates (Fukunaga and Hunter, 1997). MNK1, one of the ERK1 substrates, was subsequently validated to be an in vivo substrate of ERK1 based on phosphorylation confirmed with natural immunoprecipitated ERK1.

OsMPK5 belongs to group A of rice MAPKs families with the TEY phosphorylation motif (Rohila and Yang, 2007). The OsMPK5 gene was shown to be spliced into two transcripts *OsMPK5a* and *OsMPK5b*, and only OsMPK5a possess kinase activity (Xiong and Yang, 2003). The *OsMPK5a* expression and its kinase activity are up-regulated by ABA and stress treatments (e.g., drought, salt, wounding, etc) at the transcriptional and posttranslational levels. Our previous studies showed that overexpression of *OsMPK5* resulted in enhanced tolerance to abiotic stresses such as drought, salt and cold treatment. Interestingly, suppression of *OsMPK5* expression in rice via RNA interference compromises abiotic stress tolerance, but increases PR gene expression and host resistance to pathogen infection. However, it is still unknown what proteins are targeted by OsMPK5 and how it modulates biotic and abiotic stress tolerance. Therefore, screening and identification of OsMPK5 substrates is essential for us to

further understand the role of OsMPK5 in regulating the downstream signaling pathway(s) and plant stress responses.

Here we analyzed OsMPK5 kinase activity *in vitro* and successfully performed an *in-situ* solid phase phosphorylation screen with OsMPK5 kinase. Furthermore, we chose one putative substrate OsCDPK5 and confirmed the phosphorylation event between OsCDPK5 and OsMPK5.

2.3 Results

2.3.1 OsMPK5 kinase activity is detectible *in vitro*

To conduct the *in-situ* solid phase phosphorylation, we first examined OsMPK5a

kinase activity *in vitro* to see whether the kinase activity is detectible. These include the

kinase assays in solution and solid phases. For the kinase assay in solution, we purified

the His-tagged OsMPK5a recombinant protein from *E.coli*, and tested OsMPK5a kinase

activity in reaction buffer for its ability to phosphorylate myelin basic protein (MBP), a

commonly used kinase substrate. The phosphorylational reaction was performed at room

temperature and the resulting product was separated by SDS-page gel. OsMPK5a kinase

activity was detected by checking the radioactive labeling of separated proteins. Our

results indicated that OsMPK5a was not only autophosphorylated, but also capable of

phosphorylating MBP (Figure 2.1A). However, we did not observe OsMPK5b

autophosphorylation or its ability to phosphorylate MBP *in vitro*. This result is consistent

with our previously published data (Xiong and Yang, 2003). To examine the ability of

OsMPK5 for phosphorylating MBP in solid phase, we applied different amounts (100,

500ng) of the MBP protein onto nitrocellulose membranes, mimicking the conditions of

in-situ solid phase phosphorylation screen. The membranes were blocked by treatment

with bovine serum albumin (BSA) and pre-incubated with cold ATP to remove the non-

specific phosphorylation and then incubated with the purified OsMPK5 kinase in the

presence of [γ-32P] ATP to allow the solid-phase phosphorylation. The results showed

that the phosphorylation of MBP by OsMPK5 was detectible in the solid phase even at an

amount of 100ng (Figure 2.1B). Therefore, it is practically feasible to use the

recombinant OsMPK5 for the *in-situ* solid phase phosphorylation screen.

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2.3.2 Improvement of OsMPK5 kinase activity

The previous experiments showed that the recombinant OsMPK5 kinase has enough activity to phosphorylate MBP on membranes *in vitro*. However, to screen all the potential phosphorylation substrates of OsMPK5, it is necessary to increase the potential activity of OsMPK5 kinase. We did this by increasing the expression of recombinant OsMPK5 and modifying the conditions for the kinase assay. First, we examined the expression of the recombinant OsMPK5 with different IPTG concentrations and induction temperatures. We chose to induce OsMPK5 protein expression at 0.8 mM IPTG at 30 ºC for 6 hours, where OsMPK5 expression peaks (data not shown). Second, the natural structure of the kinase protein can be destroyed during the purification process, which notably affects not only the protein kinase activity but also its functional specificity. To maintain the kinase activity, only soluble recombinant OsMPK5 was purified using a process suitable for isolation of natural proteins (Invitrogen). In addition, we found that imidazol present in the elution solution might abolish OsMPK5 kinase activity. In order to eliminate its negative effect on OsMPK5 kinase activity, we removed imidazole and concentrated OsMPK5 from the eluent using column centrifugation (Amico, Millipore). To examine the kinase activity of the OsMPK5 protein purified by this approach, we conducted *in vitro* kinase assay in solid phase with the same aforementioned method. The kinase assay demonstrated that the ability for OsMPK5 to phosphorylate MBP was significantly increased (Figure 2.2).

2.3.3 Solid phase phosphorylation screen

To carry out the *in-situ* solid phase phosphorylation, we first prepared

nitrocellulose filters containing recombinant proteins from a rice cDNA expression

library (Figure 2.3). Each filter was estimated to contain 1 x 10^4 recombinant proteins

expressed from the stress-induced rice cDNA library. A total of 20 nitrocellulose filters

were screened with the recombinant OsMPK5 kinase in the presence of [γ-32P] ATP.

The primary screen yielded 23 positive clones that usually exhibited weak radioactive

signals due to a low amount of expressed proteins (Figure 2.4A). These cDNA clones

were individually picked up and subjected to the second round of the solid-phase

phoshorylation screen. The secondary screen further confirm that many of the primary

clones were indeed phosphorylated by OsMPK5 *in vitro*, based on the understanding that

the secondary screen should evenly display many positive spots with stronger radioactive

signals (Figure 2.4B). After the secondary screening, we isolated a total of 14 positive

cDNA clones. Subsequently, in vivo excision was conducted to convert the lambda ZAP

express vector into pBK-CMV phagemids. The resulting cDNAs encoding the putative

OsMPK5 substrates were sent for sequenicing.

2.3.4 Bioinformatic analyses of the OsMPK5 putative substrates

After obtaining the cDNA sequences, bioinformatics analyses were carried out

using the online genome databases provided by the National Center for Biotechnology

Information (www.ncbi.nlm.nih.gov) and The Institute for Genomic Research

(www.tigr.org; now renamed as J. Craig Venter Institute, www.jcv.org). Particularly,

translated protein sequences were analyzed for their sequence homology and potential

function. Based on the functional classification provided by the TIGR rice genome

database, the 14 independent cDNA clones encode several categories of proteins, including apoptosis, transcription factors, phosphatase, and protein kinases (Table 1). Strikingly, many of these putative substrates were previously shown to be involved in biotic and/or abiotic stress response, although their association with the MAP kinase signaling is largely unknown. This result suggests that OsMPK5 may mediate downstream stress signaling pathways by regulating protein phoshorylation and dephosphorylation, transcription, metabolism and programmed cell death. This information will also lead us to characterize some potentially important substrates and further our understanding of the OsMPK5-mediated stress signal transduction.

Due to the required specificity for activating downstream components, protein kinases phosphorylate their substrates by targeting conserved peptide sequences. All MAPKs contain the conserved kinase activity domain and phosphorylate S/TP sites of their substrates (Liu and Zhang, 2004). To identify potential phosphorylation sites, the peptide sequences of 14 putative substrates were scanned with the Eukaryote Linear Motif (ELM) program. As expected, ten out of the fourteen candidates were found to contain one or more putative phosphorylation sites and conserved motifs (Table 1).

2.3.5 Confirmation of the phosphorylation screen results by *in vitro* kinase assay

To confirm the phosphorylation screen results, one of the putative substrates, OsCDPK5, was selected for further *in vitro* phosphorylation by OsMPK5. The OsCDPK5 protein was tagged with GST and the resulting recombinant protein was purified from *E. coli* cells. Subsequently, MBP (as a positive control), GST-OsCDPK5 and His-OsMPK5 were applied in different amount (100ng, 500ng, and 1000ng) to the nitrocellulose

membranes. After pre-inculation with the cold ATP to eliminate autophosphorylation, the *in vitro* kinase assay was performed with His-OsMPK5 kinase or His-OsCDPK5 kinase, respectively. The kinase assay showed that OsMPK5 was able to phosphorylate OsCDPK5 and MBP (Figure 2.5). On the other hand, OsCDPK5 was also capable of phosphorylating OsMPK5 and MBP. However, OsMPK5 could not phosphorylate OsMPK5 on the solid-phase, and OsCDPK5 could not phosphorylate the solid-phase OsCDPK5. Our data indicate that OsMPK5 and OsCDPK5 can be phosphorylated each other, but the autophosphorylation of both kinases occurs only in single molecule but not between two molecules.

2.4 Discussion

2.4.1 Phosphorylation screen can identify putative substrates of plant MAP kinase

During the past decade, many substrates of yeast and animal protein kinases have been identified by solid phase phosphorylation screen (Hunter, 2004). To our knowledge, however, there is no published report on the use of *in-situ* solid phase phosphorylation screening for the identification of a plant protein kinase. Using recombinant OsMPK5 purified from E. coli, we have demonstrated in this study that it is practically feasible to isolate putative substrates of a plant protein kinase with a solid phase phosphorylation screen. A major issue for this biochemical screen is the fact that the *in vitro* results are not always consistent with the physiological truth. Kinase usually can phosphorylate a recognized motif with or without specific docking (Bardwell et al., 2003). Furthermore, due to the different protein folding processes between procaryote and eucaryote, a protein purified from *E.coli* may form somewhat different structures from the native one. To improve the specificity of phosphorylation screen, a number of approaches can be taken. One way is to maintain the kinase protein in the native form during the process of kinase expression and purification. Eukaryotic expression systems such as yeast and insect cells may be used to express eukaryotic proteins, which are supposed to keep kinases in natural structures that are the same as the original native forms. However, *E. coli* is still the most commonly used host due to its convenient expression system, even though the expressed proteins may be structurally different from the same proteins expressed in eukaryotes. In many cases, proteins expressed from *E. coli* and eukaryotic cells exhibit the same biochemical function (e.g., binding and phosphorylation activities). In our study, the OsMPK5 protein purified from *E. coli* retained its MAP kinase activity and was

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useful for the successful phosphorylation screening and isolation of 14 putative substrates.

Another important consideration for the *in-situ* phosphorylation screen is to decrease the non-specific phosphorylation in the phosphorylation reaction. For example, the cDNA library made with the GST fusion vector allows the expression of GST fusion proteins that can stick on the glutathione-derivatized cellulose filters, a process which reduces the binding of non-specific proteins from phages and *E.coli* (Fukunaga and Hunter, 1997). However, GST tag in some case can be phosphorylated by some kinases, which significantly increases the number of false positives. We only use a non-tag expression vector (ZAP Expression vector) to allow rice cDNA expression driven by the lacZ promoter. In addition, pre-incubation with cold ATP before solid phase phosphorylation with [γ-32P] ATP was used to remove autophosphorylation and non-specific binding of ATP. In our primary screening with recombinant OsMPK5, we did not see much background of phosphorylation with the regular nitrocellulose filters as the supporting membrane for proteins expressed phage-infected *E. coli*. Further confirmation with the secondary screen indicates our screen approach is practical feasible to identify putative substrates of plant protein kianses. As demonstrated in this study, biochemical screening often produces very useful results. However, it is necessary to validate the *in vitro* results with in vivo studies and genetic analysis

2.4.2 The putative substrates of OsMPK5 appear to be associated with biotic and abiotic stress responses

Sequence analysis and database search of 14 positive candidates reveal that the OsMPK5 putative substrates can be divided into different functional categories that

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involve cell apoptosis, ion channel, transcription, phosphorylation and dephosphoruylation. Interestingly, many of the putative substrates were previously shown in rice or other species (for their orthologues) to be associated with biotic and/or abiotic stress responses. This finding further suggests that these putative substrates may be targeted by OsMPK5 and act as downstream signaling components important for plant stress response. Brief discussion is provided below for these putative substrates that are associated with biotic and/or abiotic stress responses.

OsEIL1, the orthologue of Arabidopsis EIN3, is an ethylene responsive transcription factor (Mao et al, 2006). Phosphorylation of OsEIL1by OsMPK5 may affect OsEIL1 protein stability or OsEIL1's binding activity to other proteins or promoter regions that regulate ET-responsive gene expression and stress responses.

OsCDPK5 is a member of the calcium-dependent protein kinase family which is involved in various biotic and abiotic stress responses (Ludwig et al, 2003; Asano et al., 2005). Various environmental stimuli cause the influx of calcium, which consequently activates CDPK activity. Phosphorylation between OsCDPK5 and OsMPK5 mediate the cross-talk between the two signaling pathways (Ludwig et al. 2005).

OsMSL (MscS-like) is a family of mechanosensitive ion channel proteins. Generally, plant MSL responds to a wide variety of mechanical stimuli and regulates cell tolerance of osmotic stresses (Kloda and Martinac, 2002). Ten MscS-Like (MSL) proteins are found in the genome of the model flowering plant Arabidopsis thaliana (Nakayama et al., 2007). Ten MscS-Like (MSL) proteins are found in the Arabidopsis genome (Nakayama, Y et al. 2007). MSL2 and MSL3, along with MSC1, are implicated

in the control of organelle morphology, such as the shape and size of plastids (Haswell and Meyerowitz, 2006)

OsNDPK (nucleoside diphosphate kinase) is a key metabolic enzyme that maintains the balance between cellular ATP and other nucleoside triphosphates (NTPs). Arabidopsis NDPK2 was found to specifically interact with AtMPK3 and AtMPK6 and regulate cellular redox state and stress tolerance (Moon et al., 2003).

TAP46 is an associate protein of plant protein phosphatase 2A (PP2A), with a possible function in the chilling response (Harris et al. 1999). TAP46 interacts with PP2A (a phosphatase) and functions in numerous basic cellular processes such as metabolism, transcription, and signal transduction.

APK1, a tyrosine/serine/threonine-specific protein kinase, is encoded by two closely related genes (APK1a and APK1b) in Arabidopsis. The APK1 N-terminal portion contains the structure of N-myristoylation, suggesting that APK1 might associate with membranes and thereby contribute to signal transduction (Hirayama and Oka, 1992).

ER33 protein is responsive to ET treatment and a homologue of bHLH transcription factor. The functions of ER33 protein are still unknown but are reported to be induced by ethylene during tomato ripening (Pilati et al., 2007).

Glycin rich protein 2B is a RNA binding protein mediating cold-inducible suppression. The glycine-rich protein AtGRP2 is one of the four members of the cold-shock domain (CSD) protein family in Arabidopsis. It is characterized by the presence of a nucleic acid-binding CSD domain, two glycine-rich domains and two CCHC zinc-fingers. AtGRP2 is a nucleo-cytoplasmic protein involved in Arabidopsis development with a possible function in cold response (Fusaro et al, 2007). Expression analysis reveals

that the AtGRP2 gene is active in meristematic tissues and is modulated during flower development. Down-regulation of AtGRP2 gene in Arabidopsis alters stamen number and affects seed development.

RBP2 is an RNA-binding protein, affecting translation elongation factor Tu and protein translation (Mutsuda et al, 1999). RNA-binding proteins, involving in the synthesis, processing, transport, translation, and degradation of RNA, are emerging as multifunctional, cellular regulatory proteins (Fedoroff, 2002).

FtsH2 is an ATP-dependent metalloprotease in thylakoid membranes and degrades several chloroplastic proteins. The protein degradation by FtsH is regulated by stresses, such as high salt treatment (Silva, 2003). An Arabidopsis leaf-variegated mutant (yellow variegated2) results from loss of FtsH2 (Miura et al. 2007). In addition, FtsH2's function in chloroplasts is light-dependent and organ-specific (Lopez-Juez and Pyke, 2005).

2.5 Methods and Materials

2.5.1 Expression and purification of recombinant proteins from *E. coli*

The full length *OsMPK5* cDNA was previously cloned into the expression vector pET28a (Xiong and Yang 2003). To produce the GST-OsMPK5 fusion protein, OsMPK5 coding region was amplified by PCR with a pair of primers incorporating restriction sites (EcoR I and XhoI) and cloned into the GST fusion vector pGEX-5X-3. *His-OsEIL1* and *GST-OsEIL1* constructs were made with the same approaches using the pET28a and pGEX-5X-3. These constructs were verified by DNA sequencing. Recombinant fusion proteins were induced in *Escherichia coli* strain BL21 (DE3) pLys S at 28 ºC with 1 mM isopropyl-β-thiogalactopyranoside (IPTG). Fifty milliliters of bacterial suspension were pelleted by centrifugation. The pellets were resuspended in 700 ul of lysis buffer (1x PBS + 20 mM HEPES + 0.1% Triton-X-100, pH 7.4), and briefly sonicated on ice. After centrifugation, the supernatants were mixed with 50 ul of nickel or glutathione beads (Invitrogen and Amersham Biosciences) and rotated at 4 ºC for 2 h. Beads were thoroughly washed in washing buffer (PBS + 20 mM HEPES, pH 7.4), and the bound proteins were eluted by the addition of 30 ul elution buffer as recommended by the manufacturers (Invitrogen). Protein concentrations were determined by the Bradford assay (Bio-Rad, Hercules, CA).

2.5.2 OsMPK5 kinase activity assay

Kinase reactions were performed at room temperature respectively for 30 min (in solution) or for 60 min (in solid phase). For the kinase assay in solution, OsMPK5 was mixed with or without MBP in kinase buffer (20 mM HEPES, pH 7.4, 5 mM EGTA, 1

mM DTT, 25 mM β-glycerophosphate, 10 mM magnesium chloride, 10 nM ATP, 30 uCi

[3 Ci/umol] of [32P]γ-ATP). Kinase reactions were terminated by the addition of SDS-

PAGE loading buffer. The reaction solution was subjected to SDS-PAGE separation and

the electrical transferring to nitrocellulose membranes. The radioactive phosphate

incorporation was visualized by exposure the gel to X-ray film. For the kinase assay in

solid phase, different amounts of OsMPK5, OsCDPK5, and MBP were applied onto

nitrocellullous membranes. The loaded membranes were submerged and incubated in

primary kinase reaction buffer (20mM HEPES pH7.4, 20mM $MgCl_2$, 10mM $MnCl_2$, 1uM

DTT, 20uM cold ATP) at room temperature for 1 hour. The membranes then were

washed 3 times with the washing buffer (20mM HEPES-NaCl, pH 7.5, 10mM $MgCl_2$,

50uM Na_3VO_4, 5 mM β-glycerophosphate, 5mM NaF, 2mM DTT, 0.1% Triton X-100).

2ug/ml OsMPK5 was used in the kinase reaction buffer (20mM HEPES-NaCl (pH 7.5),

10mM $MgCl_2$, 50uM Na_3VO_4, 5 mM β-glycerophosphate, 5mM NaF, 2mM DTT, 0.1%

Triton X-100). The membrane was washed at least 6 times with washing buffer till there

is no radioactivity detectible in the running washing buffer. The membranes were

exposed to x-ray film over night at -80 ºC. The radioactivities were detected with

autoradiography.

2.5.3 Protein expression from rice cDNA library

The rice blast fungus-induced cDNA library was made previously with the lamda

ZAP Express vector (Stratagene). For protein expression from the cDNA library, 600 ul

of *E. coli* XL1 blue cells at an OD600 of 0.5 was mixed with the library suspension

containing 1 x 10^4 pfu of phages (for each 150 mm plate) and incubated for 15 minutes at

37 ºC. NZY top agar (5g NaCl, 2g MgSO$_4$.7H$_2$O, 5g yeast extract, 10g casein hydrolysate, 15g agar, add deionized H$_2$O to a final volume of 1 liter and ajust pH to 7.5 with NaOH) was melted and cooled to 55 ºC, mixed to each aliquot of the bacteria and phage mixture, and poured evenly onto a 150-mm NZY agar plate. The plates were incubated at 37 ºC for 3-4 hours without allowing the plaques to grow larger than 1-2mm. The nitrocellulose membrane (BA85, Schleicher & Schuell) impregnated with 1mM IPTG was placed on top of agar plates. The induction lasted at least 10 hours to facilitate protein expression in *E.coli*.

2.5.4 *In-situ* solid phase kinase assay

The procedure for *in-situ* solid phase kinase assay was based on the protocol described previously (Fukunaga and Hunter, 1997). After incubation, the nitrocellulose filters were peeled off and immersed in blocking solution [20mM Tris-HCl (pH 8.0), 150mM NaCl, and 3% BSA] and gently agitated at room temperature for 60 minutes. The filters were then washed three times for 20 minutes each in the wash buffer [20mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 mM EDTA, 1mM EGTA, 0.5% Triton X-100, 1mM dithiothreitol (DTT), and 0.2 mM phenylmethylsulfonyl fluoride (PMSF)] and rinsed for 10 min in MAPK reaction buffer (MRB) [20mM HEPES-NaCl (pH 7.5), 10mM MgCl$_2$, 50uM Na$_3$VO$_4$, 5 mM β-glycerophosphate, 5mM NaF, 2mM DTT, 0.1% Triton X-100]. Then, the filters were incubated for 60 minutes at room temperature in MRB containing 25 uM unlabeled ATP to mask proteins which have autophosphorylating and/or ATP-binding activities. Following washing for 10 min in the MRB without ATP, filters were incubated for 60 min at room temperature with gentle shaking in MRB (2 ml of the

solution per 137 mm filter) containing 25 uM unlabeled ATP, 5uCi/ml [γ-32P] ATP and 1ug/ml purified OsMPK5. The filters were washed six times for at least 5min in MAPK wash buffer [20mM Tris-HCl (pH 7.5), 150mM NaCl, 10mM EDTA, 1mM EGTA, 20mM NaF] containing 0.1% Triton X-100, and then once for 10 min in MAPK wash buffer without Triton X-100. The filters were air-dried and exposed to X-ray films for 12-48 hours at -80 ºC with intensifying screens. Positive clones were isolated and further verified by secondary screening

2.5.5 *In vivo* excision of phagemid from the ZAP express vector

The ZAP express vector is designed to allow in vivo excision and recircularization of any cloned insert contained within the lambda vector to form a phagemid containing the cloned insert. The in vivo excision process is conducted with the helper phage M13 protein, which recognizes and cleaves the replication origin. In this case, the complete pBK-CMV phagemid was replicated and used for sequencing the cDNA insert.

The excision procedure was carried out according to the manufacturer's instruction (Stratagene). The plaques containing positive clone were cored from the agar plate, and the phage particles were released into 500ul of SM buffer (100mM NaCl, 10mM MgSO$_4$, 50mM Tris-HCl, pH 7.5, 0.01% gelatin) containing 20ul of chloroform by vortexing and incubating for 1-2 hours at room temperature. XL1-Blue MRF' cells were grown overnight at 30 ºC in NZY broth supplemented with 0.2% (w/v) maltose and 10 mM MgSO$_4$. XLOLR cells were also grown in NZY broth at 30 ºC over night. XL1-Blue MRF' and XLOLR cells were suspended at an OD600 of 1.0 in 10mM MgSO$_4$. A

mixture containing 200ul of XL1-Blue MRF' cells at an OD600 of 1.0, 250 ul of phage stock (Containing > 1x 105 phage particles), and 1ul of the ExAssit helper phage (>1x 106 pfu/ul) was incubated at 37 ºC for 15 minutes, followed by adding 3ml of NZY broth and incubated for another 4 hours at 37 ºC with shaking. The excision process was stopped by heating the reactions at 65-70 ºC for 20 minutes. After spinning down at 1000x g for 15 minutes, the supernatant contains the excised pBK-CMV phagemid.
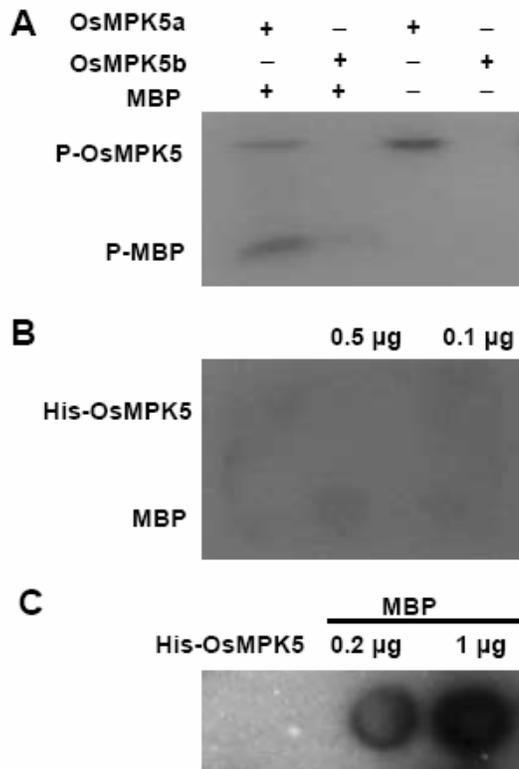
Figure 2.1 Confirmation and improvement of OsMPK5 autophosphorylation and phosphorylation activities by kinase assays.

A, The kinase assay in solution.1 ug of OsMPK5a or b was mixed with or without 1ug MBP in the kinase buffer supplemented with γ-32P-ATP. The reaction products were separated by SDS-PAGE and detected by autoradiography. B, The kinase assay in solid phase. MBP (Invitrogen) and the purified His-OsMPK5 (as a control) were applied onto the nitrocellulous membrane. The loaded membranes were pre-incubated with the cold ATP to eliminate the autophosphorylation. The kinase reaction was performed at room temperature in the reaction buffer in the presence of γ-32P-ATP with active OsMPK5a kinase. [32]P incorporation was measured by autoradiography. C, Enhanced kinase activity in solid phase based on improved procedures for OsMPK5 purification and kinase assay.
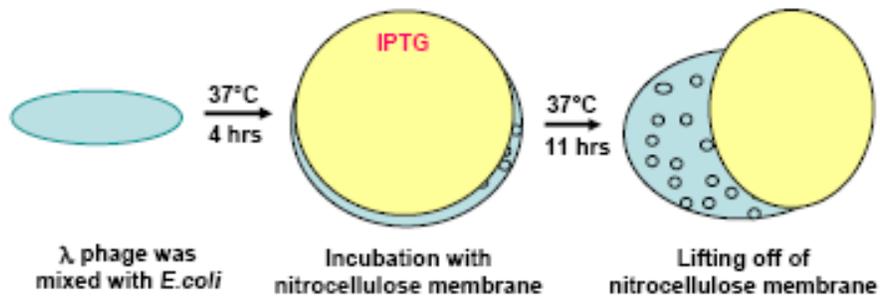
Figure 2.2 Expression of the rice cDNA library and preparation of nitrocellulose filters.

The entire process includes the attachment of the λ phage to the *E. coli* cells (XL1-Blue) by mixing proper concentration of phages and bacteria, the proliferation of both phages and cells by incubating at 37°C for 4 hours, and the induction of protein expression by incubating for another 11 hours in the presence of IPTG.
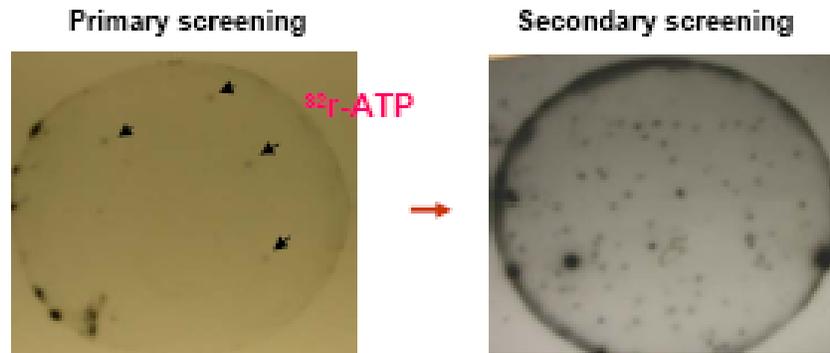
Figure 2.3 *In situ* solid phase phosphorylation screen.

The process includes the primary screen for the positive spots and the secondary screen to confirm the potential substrates of OsMPK5. Both primary and secondary screenings are cooperated with γ-32P ATP. The arrow bars indicate the positive spots which were picked individually for the secondary screening.
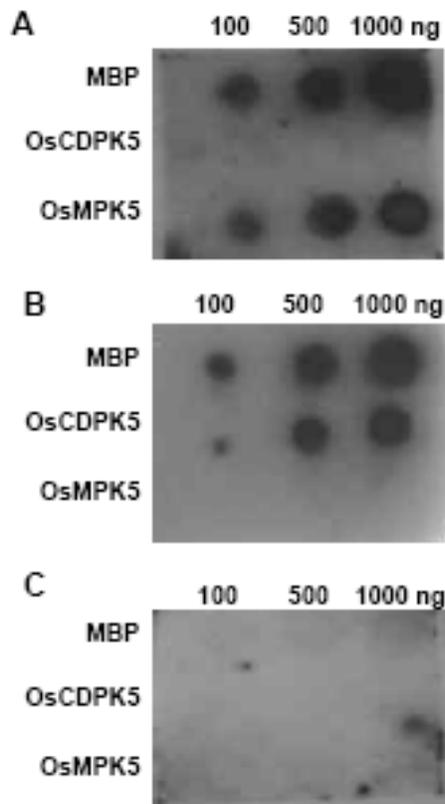
Figure 2.4 Phosphorylation between OsCDPK5 and OsMPK5 in solid phase.

The recombinant protein kinases (GST-OsCDPK5 and HIS-OsMPK5) and MBP (as a positive control) were applied onto nitrocellulose membranes with different amounts (100ng, 500ng, and 1ug). The loaded membranes were pre-incubated with the cold ATP to eliminate the autophosphorylation. The kinase reactions were performed at room temperature in the presence of γ-32P-ATP with OsCDPK5 (A), OsMPK5 (B), or no kinase (C). ^{32}P incorporation was measured by autoradiography.

Table 1 A list of the putative substrates of OsMPK5. Based on the first and the secondary screen, a total of 14 positive clones were isolated from rice cDNA library. Bioinformatic analyses reveal that these genes encode various proteins involved in protein phosphorylation, dephosphorylation, transcription, metabolism and apoptosis. Furthermore, putative MAPK phosphorylation sites for each candidate substrate were identified using the Eukaryote Linear Motif (ELM) program.

Protein ID	Protein	Phosphorylation sites (Conserved motif)	Function
NP_001047463.1	Succinyl-CoA ligase beta-chain	1 (Poor)	Ligase for GDP and ADP biosynthesis
NP_001068555.1	Ribulose bisphosphate carboxylase/oxygenase activase	1 (PAS**T**PTN)	Enzyme
NP_001060914.1	Glycine-rich protein 2b	1 (Poor)	Cold shock protein
NP_001047683.1	Mechanosensitive ion channel family protein	10 (*)	Osmotic homeostasis
NP_001053182.1	ER33 protein	1 (RNT**S**PRD)	Transcription factor
NP_001061700.1	RBP2 protein	3 (TDH**S**PAP…RAI**S**PSG…IKF**T**PPS)	Transcription regulation
NP_001049156.1	Kinase APK1B	1 (GMV**S**PED)	Kinase
NP_001055722.1	bHLH transcription factor	1 (Poor)	Transcription factor
NP_001058324.1	DNA binding protein	5 (CAV**S**PAA…MDT**S**PVI SIE**S**PST…NLT**S**PNF…NLG**S**PQS)	Transcription factor
NP-001058321.1	OsFtsH2	4 (EAI**S**PEL…AMR**T**PGF…GTL**T**PGH…PPA**T**PAA)	Protein degradation
NP-001065404.1	nucleoside diphosphate kinase 1	The docking motif with MAPK (RKEIALWF)	Enzyme
ABA95766	PP2A regulatory subunit TAP46	1 (KKL**T**PCG)	Phosphotase
AAF23901	OsCDPK5	2 (DAG**T**PGG…NVQ**T**PRS)	Kinase
AAZ78349	OsEIL1	6 (GTG**S**PHS…KHM**S**PDI…NKP**S**PPA TYN**T**PNQ…QQT**T**PAQ…RDD**T**PFG)	Transcription factor

* indicates

GKS**S**PRV…GNV**S**PNT…TAS**T**PRG…WTN**S**PRS…SPR**S**PPK…QAS**S**PSL…PSL**S**PQP…VLR**T**PTK…YYR**S**PDM…HVA**T**PV

The red color indicates the potential phosphorylation site by MAPKs

Chapter 3 OsMPK5 Phosphorylates OsEIL1 and Regulates Ethylene Signal Transduction

3.1 Abstract

MAP kinase cascades play an important role in the regulation of plant growth and development as well as biotic and abiotic stress responses. Rice mitogen-activated protein kinase 5 (OsMPK5), an orthologue of *Arabidopsis* AtMPK3, was previously found to positively regulate rice tolerance to abiotic stresses, but negatively modulate disease resistance to pathogen infection. However, the stress signaling pathway(s) mediated by OsMPK5 remains to be elucidated. In this study, we demonstrate that OsMPK5 may act as a negative regulator of ethylene signaling by impinging on signal transduction between OsEIN2 and OsEIL1 in rice. Suppression of OsEIN2 was shown to increase OsMPK5 mRNA, protein, and kinase activity. *In vitro* protein binding and kinase assays reveal that OsMPK5 interacts with and phosphorylates OsEIL1 *in vitro*. In addition, OsMPK5 protein or kinase activity is negatively correlated with the OsEIL1 protein level and downstream *OsERF1* and *OsPR5* expression in transgenic rice, suggesting that OsMPK5 mediates biotic and abiotic stress responses by negatively regulating OsEIL1 stability and ethylene signaling. Transgenic analysis showed that OsEIL1 positively regulated OsERF1 and OsPR5 expression and increased rice disease resistance against the rice blast infection.

3.2 Introduction

The plant hormone ethylene regulates a variety of developmental and stress responses in plants, including seed germination, cell elongation, fruit ripening, flower senescence, and defense against pathogens (Bleecker and Kende, 2000). In *Arabidopsis*, ethylene is perceived by a family of receptors (ETR1, ETR2, ERS1, ERS2, and EIN4) that are similar to bacterial two-component histidine kinase (Hua and Meyerowitz, 1998). ETR1 and ERS1 are localized in the endoplasmic reticulum (ER) membrane as disulfide-linked homodimers (Hall et al., 2000). The perception of ET by the receptors is followed by CTR1, a Raf-like MAP kinase kinase kinase (MAPKKK), which is physically associated with the receptors and negatively regulates downstream ET signaling events (Kieber et al., 1993). Genetic analysis in *Arabidopsis* reveals that EIN2 (ETHYLENE INSENSITIVE 2) is a central component downstream of CTR1 in the ET signaling pathway. EIN2 is a unique membrane-anchored protein and shares sequence similarity with the family of NRAMP metal ion transporter (Alonso et al., 1999). However, EIN2 itself has no demonstrated metal transporting activity. It is proposed that derepression of EIN2 by CTR1 leads to the activation of EIN3 and EIN3-like (EIL) transcription factors (Chao et al., 1997). The EIN3/EIL protein level is also modulated through proteolysis by a SCF (SKP1/Cullin/F-box protein) E3 ubiquitin ligase complex. Two *Arabidopsis* F-box protein, EBF1 and EBF2 (EIN3 BINDING FACTOR1 and 2) were shown to bind and target the constitutively produced EIN3 for degradation in the absence of ET (Guo and Ecker, 2003). In *Arabidopsis*, EIN3/EIL transcription factors recognize the EIN3-binding site located in the promoters of EREBP/ERF (Ethylene Response Element Binding Protein/Ethylene Response Factor) genes and subsequently regulate their expression.

EREBP/ERF proteins bind to the so-called GCC box present in promoters of many ET-inducible, defense-related genes and function as transcription activators or repressors (Broekaert et al., 2006).

The mitogen-activated protein (MAP) kinase cascade, which consists of at least three components, MAPKKK, MAPKK and MAPK, is involved in a plethora of signaling pathways associated with plant growth and development as well as abiotic and biotic stress responses (Nakagami et al., 2005). Among 17 rice MAPK genes, at least half of them are associated with biotic and/or abiotic stress response (Reyna and Yang, 2006; Rohila and Yang, 2007). OsMPK5, the rice orthologue of AtMPK3 and NtWIPK, was shown to positively regulate abiotic stress tolerance but negatively modulate disease resistance in rice (Xiong and Yang, 2003). Recent evidences suggest that MAP kinase cascades play a significant role in the regulation of the ethylene biosynthesis and signaling pathway (Figure 3.1). For example, mutants of *Medicago* MAPKK and MAPK (SIMKK/SIMK) exhibit the constitutive triple response phenotypes. The up-regulation of SIMKK kinase activity increased SIMK phosphorylation and mediated aminocyclopropane-1-carboxylic acid (ACC, the precursor of ET) induction (Ouaked et al., 2003). In Arabidopsis, AtMPK3/6, which are inducible by both ethylene and abscisic acid treatments, were shown to be activated by different AtMPKKs in cytosol and nuclei. In cytosol, AtMPKK4/5 and AtMPK3/6 cascades promote ethylene synthesis by phosphorylating and stabilizing ACC synthase ACS2/6 (Liu and Zhang, 2004). But in nuclei, AtMPKK7/9 and AtMPK3/6 cascades bifurcate regulation of ethylene signal transduction by positively (phosphorylating at T174) or negatively (phosphorylating at T592) regulating the stability of AtEIN3 protein (Yoo et al., 2008).

Currently, there is a major knowledge gap in ET signal transduction as to how CTR1 and EIN2 relay the signal to downstream EIN3 and EIN3-like (EIL) transcription factors. It is also unknown whether OsEIL1 is regulated by rice MAP kinase and mediates downstream EREBP transcription factors and defense gene expression. In this study, we have shown that OsMPK5 acts downstream of OsEIN2 by interacting with and phosphorylating OsEIL1. Furthermore, OsEIL1 was found to positively regulate *OsERF1* and *OsPR5* expression as well as rice disease resistance to M. oryzae infection.

3.3 Results

3.3.1 OsMPK5 expression and its protein level are negatively regulated by *OsEIN2*

We hypothesize that OsMPK5 acts downstream of OsEIN2 and regulates OsEIL1 through protein phosphorylation. If this is the case, *OsEIN2* mutation should significantly affect *OsMPK5* expression or its protein level whereas *OsMPK5* mutation should affect OsEIL1 expression and its protein level. In rice, there are two EIN2-like genes (*OsEIN2a* and *OsEIN2b*) which likely play redundant roles. As expected, suppression of *OsEIN2a*, *OsEIN2b* or *OsEIN2a+b* in rice by RNAi resulted in transgenic lines that are insensivite to ET, but hypersensitive to ABA treatment (Zhou et al., 2006; Yang, unpublished data). To examine the role of OsEIN2a and OsEIN2b on OsMPK5 expression, RNA and protein blot analyses were conducted using total RNA and proteins isolated from wild type and RNAi transgenic lines. RNA blot analysis showed that *OsMPK5* transcript was highly expressed in the *OsEIN2a+b* RNAi lines (Figure 3.1A). OsEIN2a and OsEIN2b single mutant RNAi lines also exhibited higher levels of OsMPK5 expression than those found in wildtype plants (data not shown). In comparison with wildtype plants, *OsEINa+b* RNAi lines also exhibited much higher levels of OsMPK5 protein (Figure 3.1B). By contrast, the level of OsMPK5 protein is significantly reduced in the *OsMPK5* RNAi lines. These data indicate that OsEIN2 act as a negative regulator to suppress *OsMPK5* expression in rice.

3.3.2 OsMPK5 interacts with and phosphorylates OsEIL1 *in vitro*

AtEIN3 encodes a transcription factor that acts downstream of *AtEIN2* in Arabidopsis ET signaling pathway. In rice, there are six *EIN3*-like genes. Among them,

OsEIL1 was shown to share the highest sequence identity with AtEIN3 (Figure 3.2A).

Complementation analysis demonstrated that *OsEIL1* was able to rescue the triple

response phenotype of the *AtEIN3* knockout mutant (Mao et al., 2002). Using the pull-

down protein binding assay, OsEIL1 was found to physically interact with OsMPk5. A

marked protein band of GST-tagged OsEIL1 was seen in the western blot as it was pulled

down with the His-tagged OsMPK5, whereas the GST protein control was hardly pulled

down by the His-OsMPK5 protein (Figure 3.2B). To search for potential phoshorylation

sites, the peptide sequence of OsEIL1 was analyzed with the Eukaryotic Linear Motif

program (Puntervoll, P et al., 2003). The bioinformatic analysis predicts the presence of

six putative phosphorylation sites (210S, 296S, 481S, 492T, 582T, 594T) in OsEIL1

protein. To narrow down and identify the phosphorylation region, we made three

truncated versions of OsEIL1 by deleting the putative phosphorylation sites one by one,

starting from C-terminus. Among the three truncated proteins (ΔC1, ΔC2, and ΔC3), ΔC1

(1-593) and ΔC2 (1-581) resemble the full-length OsEIL1 in which they were

significantly phosphorylated by OsMPK5 (Figure 3.2 C and D). But phosphorylation of

ΔC3 (1-491) by OsMPK5 almost disappears *in vitro*. This result suggests that the region

between 492 and 58 contains the site(s) phosphorylated by OsMPK5, and it is very likely

that T492 is the site for OsMPK5 phosphorylation. To further verify the phosphorylation

site, site-directed mutagenesis was conducted at T492 to convert threonine residue to

alanine. Subsequent kinase assay showed that the T492A mutant phosphorylation by

OsMPK5 was greatly decreased *in vitro* (Figure 3.3). These data demonstrate that

OsMPK5 interacts with OsEIL1 and phosphorylates the protein at the site of T492.

3.3.3 OsMPK5 negatively regulates the OsEIL1 protein *in vivo*

In Arabidopsis, AtEIN3 is up-regulated by a post-translational mechanism in response to ethylene treatment (Potuschak et al., 2003). To examine the regulation of OsEIL1in rice, the levels of OsEIL1 mRNA and protein in two-week old rice plants were analyzed in response to ACC treatment. RNA and protein blot analyses showed that ACC application did not significantly altered the OsEIL1 mRNA level, but drastically increased its protein level two hours after the treatment (Figure 3.4). Like AtEIN3, therefore, OsEIL1 is translationally and/or post-translationally regulated by ACC.

The carboxyl terminus of AtEIN3 is the binding region to the F box proteins EBF1/2 that recruit AtEIN3 for degradation through the proteasome-dependent process (Guo and Ecker, 2003). We hypothesized that the phosphorylation of OsEIL1 C-terminal region by OsMPK5 may affect OsEIL1 protein stability. To examine the potential regulation of OsEIL1 protein by OsMPK5, kinase activity assay and western blot analysis were carried out using proteins extracted from two-week-old rice plants of different transgenic lines (wild type, *OsMPK5* RNAi-line, *EIN2* RNAi-line, and *OsEIL1* RNAi-line). In comparison with wild-type plants, OsMPK5 autophosphorylation activity was lower in *OsMPK5* RNAi line, but higher in *OsEIN2a+b* RNAi-line (Figure 3.5A), which which was previously shown to have a higher level of OsMPK5 protein. Western blot analysis demonstrated that the *OsMPK5* RNAi line showed significantly higher level of OsEIL1 protein, whereas the *OsEIN2* RNAi line contained much lower level of OsEIL1 protein (Figure 3.5B). These results showed that OsMPK5, which is negatively modulated by OsEIN2, act as a negative regulator of OsEIl1 protein stability.

3.3.4 OsEIL1 positively regulates the expression of *EREBP* and *PR* genes

AtEIN3 is a primary transcription factor in the ethylene signaling pathway and controls the expression of a secondary transcription factor (AtERF1) which further regulates the expression of defense-related genes with the GCC box cis-element (Solano et al., 1998; Shigyo et al., 2006). In rice, OsEIL1 and OsERF1 are the orthologues of AtEIN3 and AtERF1, respectively. Exogenous treatment of wildtype plants with ACC significantly increased the expression of *OsERF1* in rice (Figure 3.6A). To determine the role of OsEIL1 in the ET-mediated transcriptional regulation, the *OsEIL1* RNAi lines were generated using the *Agrobacterium*-mediated rice transformation. As expected, the *OsEIL1* RNAi line exhibited a drastic decline of *OsEIL1* mRNA level. Interestingly, suppression of *OsEIL1* expression greatly decreased the expression of *OsERF1* and *OsPR5* genes (Figure 3.6B and C), suggesting the positive regulation of *OsERF1* and *OsPR5* expression by the OsEIL1 transcription factor. On the other hand, suppression of OsMPK5, which was shown to increase the level of OsEIL1 protein (Figure 3.5B), significantly enhanced the expression of *OsERF1* and *OsPR5* genes (Figure 3.6C). These data demonstrate that OsMPK5 negatively regulates *OsERF1* and *OsPR5* expressions by down-regulating the level of OsEIL1 protein. This result is also consistent with our previous finding that OsMPK5 negatively regulate *PR* gene expression and rice disease resistance (Xiong and Yang, 2003).

3.3.5 *OsEIL1* RNAi lines exhibit increased susceptibility to pathogen infection

Since the *OsEIL1* RNAi line exhibited reduced expression of *PR5* genes, we would like to examine the role of OsEIL1 in host resistance against rice blast infection.

Two-week-old plants of wildtype and the *OsEIL1* RNAi lines were spray-inoculated with

M. oryzae IC-18-1/2 isolate, which is moderately virulent on Nipponbare cultivar under

our experimental conditions. Six days after inoculation, host resistanc to the fungal

infection was evaluated based on disease rating, lesion number and size, and the

abundance of *M. oryzae* rRNA. In comparison with Nipponbare wildtype plants, *OsEIL1*

RNAi lines exhibited a significant increase of disease susceptibility to *M. oryzae*

infection (Figure 3.7). This result indicates that OsEIL1 positively regulates *PR* gene

expression and contribute to host resistance against the fungal infection.

3.4 Discussion

EIN2 is a central component of ET signal transduction, whose mutation in Arabidopsis leads to complete ethylene insensitivity (Alonso et al., 1999). EIN2 is a membrane-anchored protein which shares sequence similarity with the family of Nramp metal transporter. In contrast to Arabidopsis that contains a single *EIN2* gene, rice genome carries two *EIN2*-like genes which appear to have redundant functions. Suppression of *OsEIN2a* and/or *OsEIN2b* gene expression results in reduced sensitivity to ET, but increased sensitivity to ABA treatment (Jun et al., 2004; Zhou et al., 2006). EIN3, which acts downstream of EIN2, is another important component in ET signaling pathway. OsEIL1 shares the highest sequence homology with AtEIN3 and is its functional orthologue in rice (Mao et al., 2006). However, there is no evidence that EIN2 may physically interact with EIN3 or any other ET signaling components. Up to date, it is still unclear how EIN2 may relay the signal and regulate the EIN3 transcription factor for subsequent ET-mediated responses. Genetic analysis using the *OsEIN2a+b* RNAi lines reveals that the levels of OsMPK5 mRNA, protein and kinase activity are tightly controlled by OsEIN2 in rice plants. In addition, our biochemical studies show that OsMPK5 physically interacts with and phosphorylates OsEIL *in vitro*. Therefore, OsMPK5 likely acts downstream of OsEIN2 and may impinge on signal transduction between OsEIN2 and OsEIL1 in rice. We hypothesize that the OsEIN2-mediated cellular signal (e.g., ion fluctuation) may negatively regulate the MAP kinase cascade (e.g., OsMPK5), which in turn modulates the OsEIL1 protein stability via phosphorylation.

A previous study demonstrates that AtMPK3/6 (Arabidopsis orthologues of OsMPK5) can positively regulate ET biosynthesis by phosphorylating and stabilizing

AtACS2/6 (Liu and Zhang, 2004). In rice, OsMPK5 appears to act as a negative regulator of ET biosynthesis, possibly by phosphorylating and destabilizing OsACS2 which contains the conserved phosphorylation sites in its C-terminus (Baily 2007). Most recently, AtMPK3/6 activated by AtMPKK7/9 were found to phosphorylate AtEIN3 and bifurcate EIN3 protein stability in the nucleus (Yoo et al., 2008). In this study, we have shown that OsMPK5 was able to interact with and phosphorylate OsEIN3 *in vitro*. Furthermore, in vivo OsEIL1 protein level was found to be negatively regulated by OsMPK5 protein and its kinase activity. Suppression of OsMPK5 expression significantly increased the OsEIL1 protein level without altering the OsEIL1 transcript level. Therefore, the MAP kinase cascade may not only regulate the ET biosynthesis, but also modulate the ET signal transduction in both *Arabidopsis* and rice. However, the regulatory mechanisms and subsequent biochemical and physiological effect may differ between Arabidopsis and rice.

The effect of OsMPK5 phosphorylation on OsEIL1 stability indicates that the regulation of OsEIL1 may be far more complex than what we previously thought. AtEIN3 is targeted by two F box proteins EBF1/2 for the proteosome-dependent degradation (Binder et al, 2007). It is possible that phosphorylation of OsEIL1 by OsMPK5 may alter the binding of OsEIL1 to EBF1/2 and increase the OSEIL1 degradation. In addition, in-gel kinase assay reveal that at least three other rice kinases are capable of phosphorylating OsEIL1 (data not shown). The biochemical and physiological effects of OsEIL1 phosphorylation by these kinases remain to be characterized.

Ethylene is an important modulator of disease resistance and susceptibility (Broekaert et al., 2006; van Loon et al., 2006). Depending on the plant-pathogen combination and specific environmental conditions, ET may play a positive or negative role in disease resistance. It has been proposed that flood or hypoxia-induced ET biosynthesis in rice is critical for conferring field resistance to the rice blast disease in the Southern U.S (Singh et al., 2004). Iwai et al. (2006) also observed a significant increase of ET emission in the resistant interaction at 48 hours post-infection with *M. oryzae*. Treatment of rice seedlings with aminooxyacetic acid, an ACC synthase inhibitor, suppresses the ET emission and increases rice blast lesion size and disease susceptibility. Our lab has previously demonstrated that OsEIN2b can positively regulate *PR* gene expression and rice disease resistance against *M. oryze* infection (Zhou et al., 2006). By contrast, OsMPK5 is a negative regulator of *PR* gene expression and disease resistance (Xiong and Yang, 2003). In this study, we further demonstrate that the roles of OsEIN2 and OsMPK5 are likely mediated, at least partially, by OsEIL1, which positively regulating the downstream *OsERF1* transcription factors and *OsPR5* gene (Figure 3.6). Suppression of OsEIL1 in rice by RNAi was shown to enhance disease symptoms and increase the fungal growth in planta. Since rice blast resistance appears to require both ET biosynthesis and signal transduction, a better understanding of the ET pathway should help improve breeding strategies and cultural practices for controlling rice blast disease.

3.5 Materials and Methods

3.5.1 Gene cloning and expression of OsEIL1 in *E. coli*

The full-length cDNA of OsEIL1 was amplified by the polymerase chain reaction using a specific pair of primers containing appropriate restriction sites. The amplified cDNA fragment was cloned into the expression vectors pET28a and pGEX-5X-3, respectively. His-OsEIL1 and GST-OsEIL1 proteins were expressed and purified from *E.coli* BL21 cells according to the manufacturer's instructions (Pierce and GE Health Sciences).

3.5.2 OsEIL1 Antibody

A cDNA fragment spanning from the translation start site to the 834th bp of *OsEIL1* gene was inserted in frame into the pET-28a vector at the restriction sites BamHI and XhoI. After confirmation with DNA sequencing, the recombinant constructs were introduced into the *E.coli* BL21 cell for protein expression. The His-tagged 278 aa N-terminal region of OsEIL1 was purified with a nickel column (Pierce) and used for generating the polyclonal antibody. The anti-OsEIL1 antiserum was produced by immunizing rats (antibody service provided by Cocalico Biologicals, Reamstown, PA).

3.5.3 Western blot analysis

Whole protein lysate were prepared by homogenizing leaf powders (grounded with liquid nitrogen) in the lysis buffer (20mM Tris-HCl pH7.5, 100mM NaCl, 0.1% Triton-X100, 0.2mM PMSF). Protein concentrations were determined by spectrophotometer using Bradford reagent (BioRad). Total proteins were separated on the

10% SDS-PAGE and transferred onto PVDF membranes at 10V for one hour with Trans-Blot SD Semi-Dry Transfer Cell (BioRad). PVDF membrane carrying the protein was then blocked overnight with 5% powder milk in the blocking buffer (20mM Tris-HCl, pH8.0, 150 mM NaCl, 0.1% Triton-X100), and followed with three times washing with the washing buffer (20 mM Tris-HCl, pH8.0, 150 mM NaCl, 0.1% Triton-X100) for 15 minutes each. Membranes were then probed with a primary antibody (anti-OsEIL1 or anti-OsMPK5) for one hour at room temperature. After washing three times with the washing buffer for 15 minutes each, PVDF membrane was incubated with the secondary antibody (anti-rat IgG conjugated with horse radish peroxidase, 1/10000 dilution) in the washing buffer for 30 minutes at room temperature, and followed by three times wash with washing buffer. The probed protein on the membrane was detected with the ECL chemiluminescent substrate reagent kit (PIERCE) by exposure to an X-ray film.

3.5.4 Protein binding and *in vitro* kinase assays

His-OsMPK5 and GST-OsEIL1 proteins were purified from E.coli BL21 with nickel chelated agarose beads (Pierce) and glutathione Sepharose 4B beads (Amersham Biosciences), respectively. Protein binding assay was performed at 4 ºC by mixing 0.5ug His-OsMPK5 and 0.5ug GST-OsEIL1 and incubating in the binding buffer (20mM HEPES, pH 7.4, 150mM NaCl, 1mM DTT) with slow rotation for 2 hours. The mixture was washed at least 4 times in washing buffer (20mM HEPES, pH 7.4, 1xPBS). Proteins were then separated in 10% SDS-PAGE and detected with OsMPK5 antibody and GST antibody using the SuperSignal West Pico Chemiluminescent Substrate (PIERCE). For *in vitro* kinase assay, 1ug His-OsMPK5 and 1 ug GST-OsEIL1 was mixed in the kinase

assay buffer (20mM Tris-HCl pH7.4, 20mM $MgCl_2$, 10mM $MnCl_2$, 2uM DTT) incorporated with [r-^{32}P] ATP and incubated at room temperature for 30 minutes. The kinase reaction was terminated by addition of protein loading buffer. After separation with 10% SDS-PAGE and dried with a vacumn gel dryer, the radioactivity incorporated into the protein substrate was detected by exposure to an X-ray film.

3.5.5 RNA blot and RT-PCR analyses

Total RNAs were isolated from leaf tissues of two-week-old rice plants using the Trizol reagent (Invitrogen). For RNA-gel blot analysis, 20ug total RNAs were separated with 1.2% agarose gel and transferred to nylon membranes. The hybridization was performed overnight using [α-^{32}P] CTP labeled probes in the PerfectHyb solution (Sigma) at 60 ºC. RNA blots were washed twice in 2x SSC for 15 min at 60 ºC, then twice in 1x SSC, 0.5% SDS for 20 min at 60 ºC, and finally in 0.1x SSC at room temperature for 10 min. The DNA probes for *OsMPK5*, *OsEIL1*, *OsERF1*, *OsPR5*, and *M. oryzae rDNA* were prepared using restriction enzyme digestion or PCR amplification, and radioactive labeled using random priming. Below are some specific primers using for PCR amplification: *OsEIL1* forward primer, 5'-AAG GAT ACA GAT TGA AGA AGG C-3' and reverse primer, 5'- CAT TGA TGT CGC CCA TCT GGT-3' (DQ153245; Mao et al, 2006); *OsERF1* forward primer, 5'-GCA AGC CTA CCA AGC ACA TG-3' and reverse primer, 5'-TGG AGG AAG AAG AGG AGG AG-3' (AK241281); *OsPR5* forward primer, 5'-ACC TCT TCC GCT GTC CTC-3' and reverse primer, 5'-GAA GAC GAC TTG GTA GTT GC-3' (X68197; Mei et al, 2006).

Semi-quantitative RT-PCR analysis for the OsERF1 expression was conducted using the one step RT-PCR kit (Qiagen) according to the manufacturer's instructions. Reverse transcription was carried out at 42 ºC for 2 hours using 2 ug of total RNA, followed by thirty cycles (95 ºC for 1 min, 59 ºC for 35 s, 72 ºC for 1 min) of PCR amplification. Gene-specific primers (forward: 5' GCA AGC CTA CCA AGC ACA TG 3'; reverse: 5' TGG AGG AAG AAG AGG AGG AG 3') were used to amplify the 150 bp fragment from the 3' region of OsERF1. RT-PCR experiments were repeated at least twice to validate the results. In addition, RT-PCR for the rice ubiquitin gene was performed under the same conditions as described above to determine the equal loading of the total RNA samples.

3.5.6 Chemical treatment and pathogen inoculation

Rice cultivar Nipponbare (*Oryza sativa* spp. japonica) were used in this study. For chemical tr

eatments, leaves of two-week-old rice plants were sprayed with ACC (0.1 mM) or ABA (0.1 mM) solution. For pathogen infection, two-week-old plants of wild-type control and *OsEIL1* RNAi transgenic lines were spray-inoculated with *M. oryzae* IC17 18/1-2 (moderately virulent on Nipponbare cultivar) at a concentration of 400,000 spores per ml. After incubation in a plastic bag (22°C) for 24 h, plants were moved to a growth chamber and maintained at 28°C under 16 h of light (Lee et al, 2001). Disease evaluation was conducted at six days post inoculation by measuring blast lesion size and number as well as disease rating (Marchett et al., 1976). In addition, the fungal growth *in planta* was

estimated based on the rRNA amount of *M. oryzae* revealed by RNA blot analysis (Qi

and Yang, 2000).
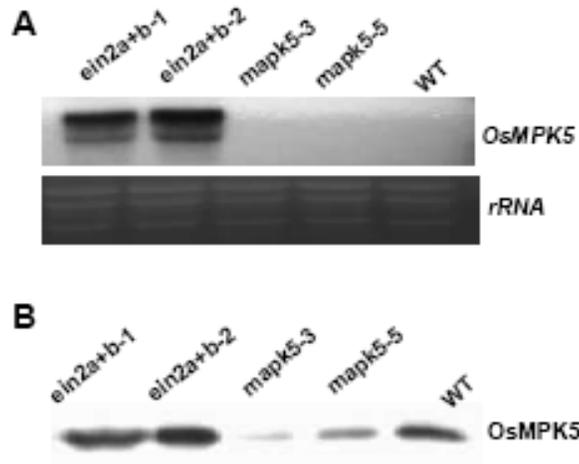
Figure 3.1 Expression of OsMPK5 mRNA and protein is negatively regulated by OsEIN2.

A, Detection of *OsMPK5* mRNA levels by RNA blot analysis; B, Detection of OsMPK5 protein levels by western blot analysis. Total RNAs and proteins were isolated from leaf tissues of various rice lines (*ein2a+b-1* RNAi line, *ein2a+b-2* RNAi line, *OsMPK5-3* RNAi line, *OsMPK5-5* RNAi line, and wild type).

A

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  1 MMGGGLVMDQGMMFPGVHNFVDLLQQNGGDKNLGFGALVPQTSSGEQCVMGEGDLVDPPP
 61 ESFPDAGEDDSDDDVEDIEELERRMWRDRMKLKRLKELQLSRGKDPAGGVVGDPSKPRQS
121 QEQARRKMSRAQDGILKYMLKMMEVCRAQGFVYGIIPEKGKPVSGASDNLRGMWKEKVR
181 FDRNGPAAIAKYQADNAVPGFESELASGTGSPHSLQELQDTTLGSLLSAIMQHCDPPQRR
241 YPLEKGVPPPWWPTGDEEWWPELGIPKDQGPPPYKKPHDLKKAWKVSVLTAVIKHMSPDI
301 EKIRRLVRQSKCLQDKMTAKEISTWLAVVKQEEELYLKLNPGARPPAPTGGITSAISFNA
361 SSSEYDVDDCKGDEAGNQKAVVVADPTAFNLGAAMLNDKFLMPASMKEEATDVEFIQKRS
421 ASGAEPEIMLNNRVYTCHNVQCPHSDYGYGFLDRNARNSHQYTCKYNDPLQQSTENKPSP
481 PAIFPATYNTPNQALNNLDFGLPMDGQRSITELMNMYDNNFVANKNLSNDNATIMERPNA
541 VNPRIQIEEGFFGQGSGIGGSNGGVFEDVNGMMQQPQQTTPAQQQFFIRDDTPFGNQMGD
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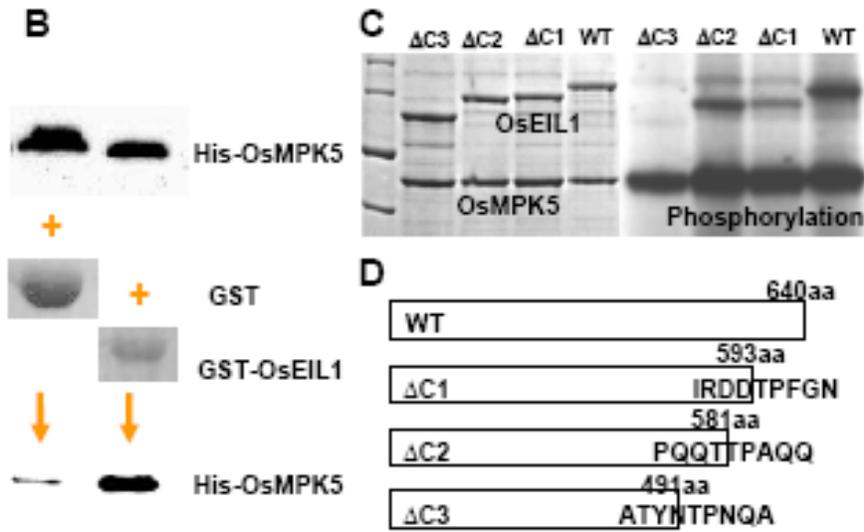
Figure 3.2 OsMPK5 interact with and phosphorylates the C-terminal region of OsEIL1.

A, Amino acid sequence of OsEIL1 protein. Red color indicates the putative phosphorylation sites by MAPK. * T492 is the phosphorylation site confirmed by the site-directed mutation and *in vitro* kinase assay (see Figure 3.4). B, *In vitro* binding of OsMPK5 with OsEIL1. The protein binding assay was conducted by mixing His-OsMPK5 with GST-OsEIL1 or GST (control) in the protein binding buffer followed by incubation for 2 hours at 4 ºC. The glutathione-agarose beads were used to pull down GST or GST-tagged proteins. After the 10% SDS-page gel separation, a polyclonal antibody against OsMPK5 was used to detect His-OsMPK5, the GST monoclonal antibody (Cocalico Biologicals) was used to detect GST and GST-OsEIL1. C, Phosphorylation of OsEIL by OsMPK5 *in vitro*. His-OsMPK5, His-OsEIL1 and its truncated vertions (ΔC1, ΔC2, and ΔC3) were purified from E.coli. 2ug OsMPK5 was mixed respectively with 2ug OsEIL1 (WT) or its truncated versions in the kinase buffer supplemented with γ-32P-ATP. ^{32}P incorporation was measured by X-ray film, and the equal protein loading was estimated based on Coomasie-blue staining. D, The diagram of the full length and the truncated versions of OsEIL1.
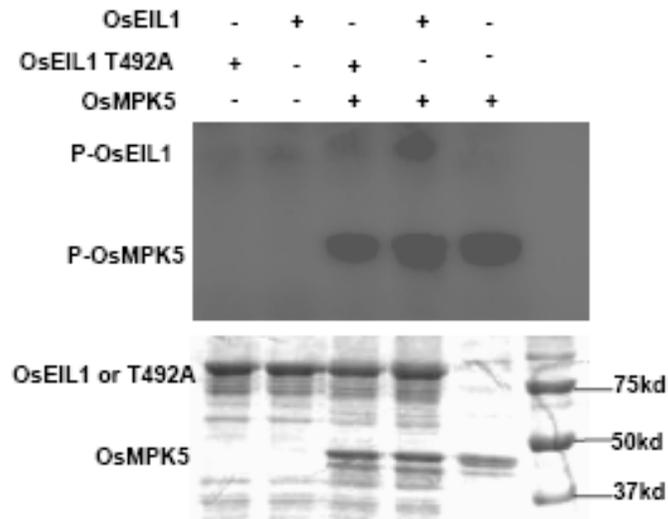
Figure 3.3 Determination of T492 as a key phosphorylation site.

OsEIL1 T492A mutant is generated using the site-directed mutagenesis. 1ug of OsMPK5 and 3ug of OsEIL1 or OsEIL1 T492A were used for the kinase assay. The kinase reaction was performed at room temperature for 30 minutes and followed by 10% SDS-gel separation. The gel was stained with Coomasia blue, dried and subjected to autoradiography.
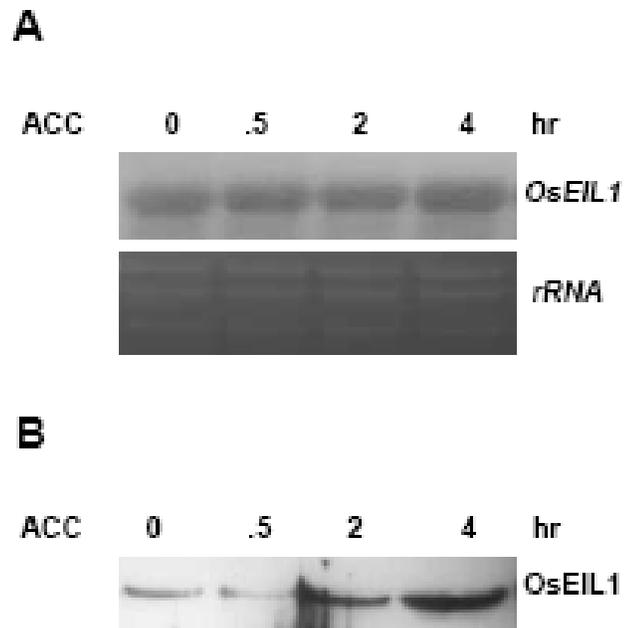
Figure 3.4 OsEIL1 protein level is up-regulated by exogenous ACC treatment.

A, Detection of *OsEIL1* mRNA level by RNA blot analysis; B. Detection of OsEIL1 protein by western blot analysis. Total RNAs and proteins were isolated from leaf tissues of two-week-old rice plants (wild type Nipponbare) treated with 0.1 mM ACC.

A

MPK5 RNAi   WT   EIN2 RNAi

P-OsMPK5

IgG 50kd

B

MPK5 RNAi   WT   EIN2 RNAi
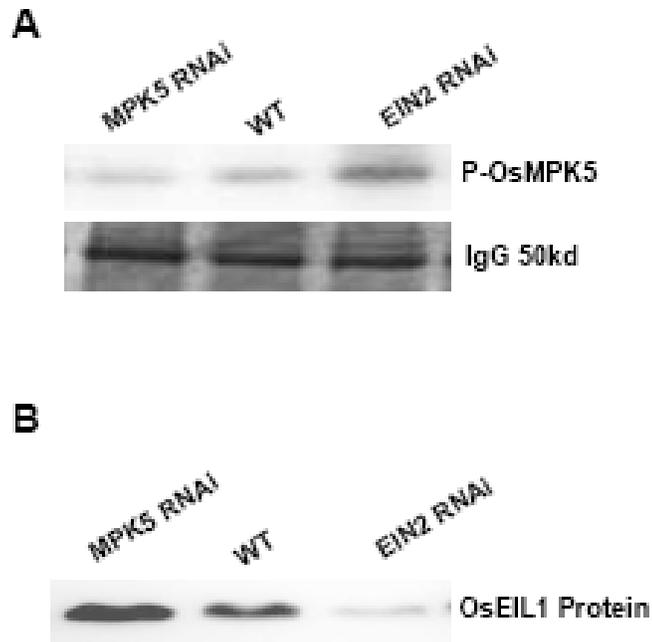
OsEIL1 Protein

Figure 3.5 OsMPK5 negatively regulates OsEIL1 protein level in vivo

A, Autophosphorylation of the natural OsMPk5. Total proteins were isolated from leaf tissues of rice lines (*OsMPK5* RNAi-line, wild type, and *EIN2* RNAi-line). Immuno-precipitation was conducted using anti-OsMPK5 antibody to isolate OsMPK5 protein from the total protein. B, Detection of OsEIl1 protein levels in various rice lines by western blot analysis.
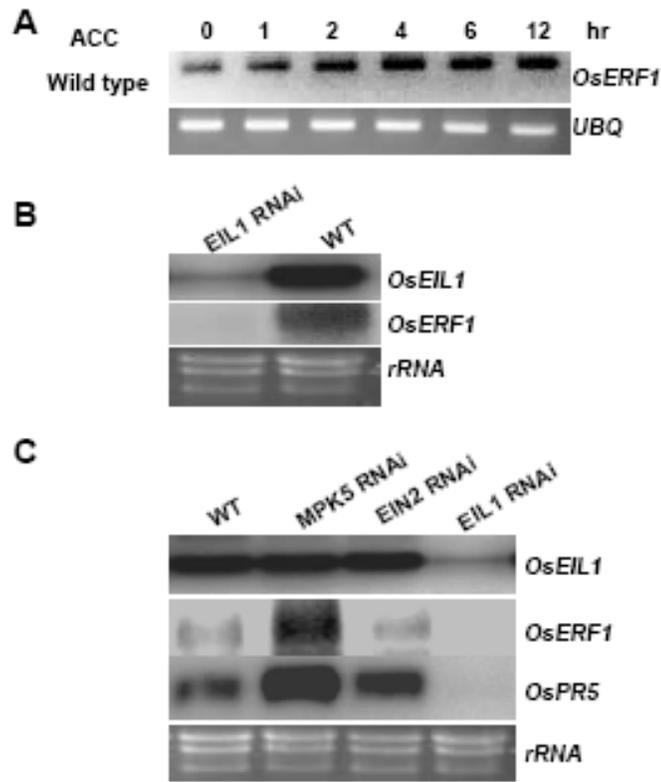
Figure 3.6 Expression of ethylene responsive genes in various rice RNAi lines.

A, Induction of *OsERF1* expression by ACC. Total RNAs were isolated from leaf tissues of wild-type Nipponbare plants treated with 1 mM ACC. The *OsERF1* mRNA levels were detected by semi-quantiative RT-PCR analysis. B, Positive regulation of *OsERF1* expression by *OsEIL1*. Total RNAs were isolated from leaf tissues of two-week-old rice lines (wild type and *OsEIL1* RNAi line) and analyzed by RNA blot analysis. C, negative regulation of ET-responsive genes by OsMPK5. Total RNAs were isolated from leaf tissues of rice lines (wild type, *OsMPK5* RNAi line, *EIN2* RNAi line, and *EIL1* RNAi line) and probed with the gene-specific fragments of *OsEIL1*, *OsERF1*, and *PR5* using RNA blot analysis.
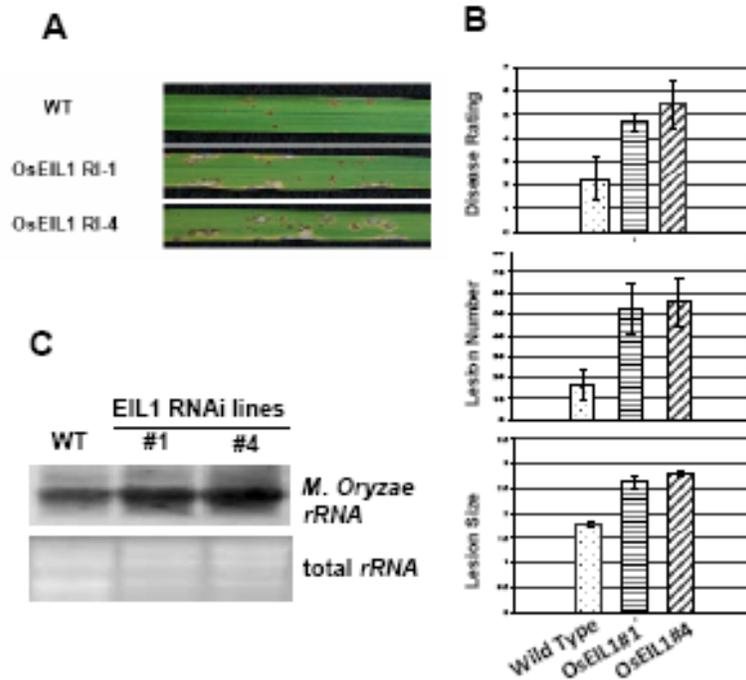
Figure 3.7 *OsEIL1* RNAi lines exhibited increased susceptibility to the rice blast fungus.

A, Disease symptoms on leaves of wild type and RNAi lines at six days after the infection of *M. oryzae* isolate IC- 18-1/2. B, Resistance evaluation based on the disease rating, lesion number and lesion size (the three largest ones) at 6 days after inoculation. Error bars indicate SD. (Student's t test; P value <0.01). C, Estimation of the pathogen growth *in planta* based on the fungal *rRNA* amount. Total RNAs from the infected leaves at 6 days after inoculation were hybridized with *M. oryzae* 25S rDNA.

Chapter 4   OsCDPK5 Interacts with and Phosphorylates OsMPK5 and Potentially Affects Rice Biotic and Abiotic Stress Tolerance

4.1 Abstract

Calcium-dependent protein kinases (CDPKs) belong to a large family of protein kinases with various roles in plant growth and development as well as biotic and abiotic stress responses. The expression of CDPKs and their kinase activities are frequently activated by various environmental stimuli such as drought, salt, cold and pathogen infection. However, it is still not very clear how CDPKs activate downstream signaling components and regulate various biotic and abiotic stress responses. Our biochemical screening identified a rice CDPK (OsCDPK5) which interact with and phoshorylate OsMPK5. The autophosphorylation activity of OsCDPK5 as well as its ability to phosphorylate OsMPK5 were regulated by calcium concentration *in vitro*. Furthermore, OsCDPK5 was found to physically interact with OsMPK5 *in vitro* and in vivo. RT-PCR and RNA blot analyses showed that the expression of *OsCDPK5* in rice was up-regulated by ACC and wounding but down-regulated by ABA. To determine the potential role of *OsCDPK5* in plant stress tolerance, transgenic RNAi lines were generated via the *Agrobacterium*-mediated rice transformation. Similar to the silencing effect of OsMPK5, suppression of OsCDPK5 enhanced the expression of *OsPR5*, suggesting that OsCDPK5 may potentially regulates rice biotic and abiotic stress tolerance.

4.2 Introduction

Like the MAP kinases, calcium-dependent protein kinases (CDPKs) belong to
another important family of protein kinases which are also involved in various biotic and
abiotic stress responses (Cheng et al., 2002; Hrabak et al., 2003). The expression and
kinase activity of CDPKs are known to be regulated by various plant hormones and
environmental stressess. In addition, suppression of CDPK expression often results in
alteration in plant development and/or stress tolerance (Leclercq et al., 2005; Li et al.,
2008; Argueso et al., 2007).

In plants, three major classes of Ca^{2+} binding proteins have been characterized,
including calcium-dependent protein kinases (CDPKs), calmodulins and calcineurin B-
like proteins (Yang and Poovaiah, 2003). Among these classes, CDPKs are calcium-
activated serine/threonine protein kinases with a highly conserved structure containing
both calcium sensors (EF hands) and effectors (kinase regions). As an important
secondary signal, calcium directly regulates the molecular structure and kinase activity of
CDPKs. The N-terminal half (kinase and autophosphorylation region) of the protein is
directly tethered via an autoinhibitory junction domain to a regulatory calmodulin-like
domain, which usually contains four functional EF hands for calcium binding (Hrabak et
al., 2003). Almost all rice CDPKs possess four EF hands in the C-terminal region, except
OsCPK5 and OsCPK25, which have three EF hands, and OsCPK6, which has only one
EF hand. Isoform-specific differences are mainly restricted to the N-terminal variable
domain (Christodoulou et al., 2004). Usually cytosolic CDPKs are in the autoinhibitory
status because of the low calcium concentration. After the Ca^{2+} influx and binding, which
is elicited by various stimuli, the conformation of CDPKs changes as the EF hand flips

back and interacts with kinase domain for the loop formation, which switches CDPKs'

kinase activities from the inhibiting status into the activated status (Cheng et al., 2002).

The Ca^{2+} signal (e.g., a change of intracellular calcium concentration) acts as an

important second messenger during the plant stress responses. Calcium concentrations in

cytosol are often influenced by various external stresses (such as salt, high and low

temperature, and pathogen infection) and plant hormones (such as abscisic acid,

gibberellic acid, cytokinin, auxin, brassinolides, jasmonic acid and ethylene) (Harper and

Harmon, 2005). The exogenous Ca^{2+} or the influx of Ca^{2+} can alter plant tolerance to

enviromental stresses. The expression of rice CDPKs is also altered in response to a

variety of exogenous stimuli. For example, the expression of *OsCDPK7* (i.e., *OsCPK13*)

was increased by cold or salt treatment (Saijo et al., 2000). Overexpression of OsCDPK7

in rice led to enhanced tolerance to cold, salt and drought stresses (Saijo et al., 2000). In

addition, the expression of OsCPK5 was induced by avirulent and virulent isolates of the

rice blast fungus (Asano et al., 2005). Komatsu et al (2004) reported that the enzymatic

activity of rice CDPKs was also induced by probenazole treatment. Because probenazole

(3-allyloxy-1, 2-benzisothiazole-1, 1-dioxide) is regarded as an effective chemical against

rice blast disease, OsCDPKs likely play a role in rice blast resistance (Ludwig et al.,

2005). However, the mechanisms underlying the CDPK-mediated biotic and abiotic stress

tolerance in plants remain to be elucidated.

A genome-wide analysis reveals a total of 29 rice *CDPK* genes that can be

divided into four distinct classes (Asano et al., 2005). All *OsCDPKs*, except for

OsCPK25 and *OsCPK26*, are detectable at the transcript level in rice plant. RNA blot

analysis indicates that *OsCPK1, OsCPK3* and *OsCPK24* are predominantly expressed in

the basal parts of the rice plants; *OsCPK9* is predominant in the leaf blade; *OsCPK10* is

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mostly expressed in the root; *OsCPK21* is expressed in the immature seed; *OsCPK20* in both the leaf blade and the basal parts; and *OsCPK17* in all tissues (Asano et al., 2005). The tissue-specific expression implicates that each OsCDPKs may play specific roles in rice growth and development.

It was previously shown that the MAP kinase cascade may cross talk with the CDPK signal pathway. However, there is no report of the direction interaction between a MAPK and a CDPK. In this study, we demonstrated that OsCDPK5 was capable of phosphorylating OsMPK5 and physically interacted with OsMPK5 *in vitro* and *in vitro*. Like OsMPK5, the expression of OsCDPK5 (OsCPK18) was regulated by ABA, ACC and wounding. Transgenic analysis using its RNAi lines indicates that OsCDPK5 negatively regulates OsPR5 expression and likely plays a role in rice biotic and abiotic stress tolerance.

4.3 Results

4.3.1 The expression of *OsCDPK5* is regulated by ABA, ACC and wounding

The *OsCDPK5* gene was initially isolated from the rice cDNA expression library following the solid phase phosphorylation screening. The *OsCDPK5* cDNA is 1828 bp long and encodes a predicted protein of 512 amino acids with an estimated molecular mass of 57.6 kD (Figure 4.1). The OsCDPK5 protein contains the conserved kinase domain and four EF hand domains. OsCDPK5 is closely related to OsCDPK1, both of which share the identical amino acid sequence in the kinase domain and an overall sequence identity of 80%.

The expression of plant CDPKs and their kinase activity are often regulated indirectly by endogenous and exogenous stimuli, including plant hormones (Ludwig et al., 2004). As mentioned previously, OsCDPK5 was identified using OsMPK5 during the phosphorylation screening. To examine the expression pattern of *OsCDPK5* in response to plant hormone and wounding treatments, RT-PCR and RNA blot analyses were conducted using total RNAs extracted from two-week-old rice plants treated with ABA, ACC and wounding. Upon ABA treatment, the *OsCDPK5* mRNA level was progressively decreased (Figure 4.2). By contrast, ACC treatment was shown to up-regulate the *OsCDPK5* mRNA level. Wounding also induced the *OsCDPK5* mRNA level within one hour and the transcript level peaked at 4 hours after treatment. Since wounding treatment typically triggers the JA pathway (Rakwal and Agrawal, 2003), OsCDPK may be also associated with the JA signaling in rice.

4.3.2 OsCDPK5 phosphorylates OsMPK5 in a calcium-dependent manner

OsCDPK5 was initially isolated as a candidate of OsMPK5 substrates by an *in-situ* solid phase phosphorylation screen. To confirm the initial screen result, kinase assays in solution were performed using recombinant GST-OsCDPK5 and His-OsMPK5 proteins. OsCDPK5 was consistently shown to phosphorylate OsMPK5 *in vitro* in a calcium-dependent manner (Figure 4.3). Not only OsCDPK5 autophosphorylation activity was increased with the addition of calcium (the activity peaked at 10 mM), but also the OsCDPK5 activity to phosphorylate OsMPK5 was enhanced with increasing concentrations of calcium. In addition, we generated the truncated versions (C-terminal and N-terminal halves) of OsMPK5. Both N- and C-terminal halves of OsMPK5 showed no autophosphorylation activity. Upon addition of OsCDPK5, the C-terminal half, but not the N-terminal half of OsMPK5 was phosphorylated (Figure 4.4), suggesting the presence of the phosphorylation sites in the C-terminal region of OsMPK5. However, we have not had sufficient evidences to demonstrate that OsMPK5 is capable of phosphorylating OsCDPK5 in solution even though OsCDPK5 was initially identified as a substrate of OsMPK5 in the solid-phase phosphorylation screening.

4.3.3 OsCDPK5 physically interacts with OsMPK5 *in vitro* and in vivo

In vitro pull-down assay and in vivo co-immunoprecipitation were used to demonstrate the physical interaction between OsCDPK5 and OsMPK5. For *in vitro* protein binding assay, recombinant GST-OsCDPK5 and His-OsMPK5 proteins were expressed and purified from *E.coli*. Protein binding reaction was performed at 4 ºC and detected with a monoclonal antibody against GST and a polyclonal antibody against OsMPK5. A significant amount of His-OsMPK5 was pulled down by GST-OsCDPK5,

but not by GST only, indicating that OsCDPK5 physically interacts with OsMPK5 *in vitro* (Figure 4.5A). To further demonstrate their interaction in vivio , co-immunoprecipitation was performed using antibodies against OsCDPK5 and OsMPK5, respectively. Strikingly, both OsCDPK5 and OsMPK5 were shown to be pulled down by the OsCDPK5 antibody or the OsMPK5 antibody (Figure 4.5B). This result suggests a strong physical binding between OsCDPK5 and OsMPK5 in vivo.

4.3.4 Generation and verification of *OsCDPK5* RNAi lines

To determine the biological function of OsCDPK5 and to explore the potential relevance to the OsMPK5 function, stable RNA interference approach was used to suppress the expression of *OsCDPK5* in rice plants. A 500 bp gene-specific fragment was amplified from the 3' region of *OsCDPK5* and inserted into the rice RNAi binary vector pANDA (Miki et al., 2004). Since OsCDPK5 and OsCDPK1 are closely related (80% identity at the amino acid level) and may have redundant functions, the RNAi construct containing both gene fragments were also made for silencing the expression of both genes. Subsequently, the *Agrobacterium*-mediated rice transformation was used to generate transgenic rice plant (4.6 A). A total of 14 *OsCDPK5* RNAi lines were regenerated from transgenic calli. However, we were able to regenerate RNAi lines with suppression of both *OsCDPK1* and *OsCDPK5* expression. It is possible that suppression of both genes may negatively affect the regeneration and the formation of plantlets. To examine the expression of OsCDPK5 in the RNAi lines, RNA blot analysis was carried out using total RNAs extracted from 14 primary (T0) transgenic plants. In comparison with wildtype plants, 10 out of 14 transgenic RNAi lines showed a reduction of the

OsCDPK5 mRNA level (Figure 4.6B). Subsequently, rice seeds were harvested from the T0 RNAi lines and used for further transgenic analysis.

4.3.5 OsCDPK5 negatively regulates *OsPR5* expression and is likely involved in rice biotic and abiotic stress tolerance

Because OsCDPK5 interacts with and phoshorylates OsMPK5 and its expression is altered in response to ACC and ABA treatments, we hypothesize that OsCDPK5 may affect the OsMPK5 signaling and downstream *PR* gene expression. To test this hypothesis, the expression of *OsPR5* in wild type, *OsMPK5* RNAi line and *OsCDPK5* RNAi lines were measured with RNA blot analysis. Interestingly, the levels of *OsPR5* mRNA were significantly increased in *OsCDPK5* RNAi lines (Figure 4.7A), as in the case of *OsMPK5* RNAi line. This preliminary data suggests that OsCDPK5 may regulate OsMPK5 signaling and affect downstream OsPR5 expression. Our preliminary studies also found that *OsCDPK5* RNAi lines exhibited enhanced tolerance to salinity (Figure 4.7B) and increased susceptibility to aphid infestation (Figure 4.7C). However, further analyses using T1 and T2 transgenic plants are required to establish the role of OsCDPK5 in rice biotic and abiotic stress tolerance.

4.4 Discussion

Both the MAPK and CDPK pathways have been demonstrated to play important roles in plant biotic and abiotic stress responses. A previous report suggests that ET may mediate the crosstalk between the MAPK and CDPK pathways and influence plant stress response (Ludwig et al., 2005). However, there is no report about the direction interaction between a MAPK and a CDPK. In this study, *in vitro* protein binding assay reveals that OsCDPK5 physically interacts with OsMPK5. More importantly, the direct interaction between OsCDPK5 and OsMPK5 was verified by in vivo coimmunoprecipitation. These data strongly suggest that the crosstalk between the two pathways can be mediated by the physical interaction between a CDPK and a MAPK.

In addition to the binding, OsCDPK5 was shown to phosphorylate the C-terminal region of OsCDPK5 in a calcium-dependent manner. The autophosphorylation activity of OsCDPK5 as well as its ability to phosphorylate OsMPK5 were regulated by calcium concentration *in vitro*, with a peak activity at 10 mM Ca^{2+}. It is well known that intracellular calcium concentration is influeced by a wide range of external stimuli such as hormone treatment and biotic/abiotic stresses. For example, ABA treatment elevates calcium level in guard cell, leading to the stomatal closure. Identification of OsCDPK5 as a direct regulator of OsMPK5 provides an interesting link between calcium sensing and the MAP kinase signaling in plants.

The regulation of CDPK expressions and kinase activities by hormones has been described in various plant species (Khan et al, 2005; Zhu et al, 2007; Li et al, 2008). Although we have not yet exmined the OsCDPK5 kinase activity in response to hormone or stress treatment, we found that the mRNA of *OsCDPK5* was up-regulated by ACC and

wounding, but downregulated by ABA, suggesting the potential role of OsCDPK5 as a molecular node in response to ACC and ABA signaling. Namely, OsCDPK5 may have the potential to mediate the crosstalk between the ET and ABA signaling pathways. Nevertheless, there are many questions that remain to be elucidated. Do both ACC and ABA regulate cytosolic calcium concentrations at the specific tissues where OsCDPK5 functions? Do ACC and ABA regulate OsCDPK5 phosphorylation through activating other kinases? If OsCDPK5 phosphorylation was induced by ACC and ABA, do the phosphorylation sites of OsCDPK5 occur at the same amino acids? Clearly, more detailed molecular and biochemical characterization is required to address these questions. To elucidate the biological function of *OsCDPK5*, transgenic RNAi lines with suppressed expression of *OsCDPK5* were generated via the Agrobacterium-mediated transformation and verified by RNA blot analysis. Interestingly, suppression of OsCDPK5 resulted in an increase of *OsPR5* expression. Our previous data show that OsMPK5 phosphorylates and negatively regulates OsEIL1 and its downstream OsPR5 genes. Therefore, OsCDPK5 may regulate ethylene signaling pathways and the GCC box gene expression via the interaction and phosphorylation of OsMPK5.

Since OsCDPK5 was shown to regulate PR gene expression, it is logical to assume its potential involvement in biotic and abiotic stress tolerance. Our preliminary data show that silencing of *OsCDPK5* appears to enhance salt tolerance. We also observed severe infestation of the *OsCDPK5* RNAi by aphids. However, these preliminary results must be carefully examined and verified with additional transgenic analysis using seeds from the seond generation transgenic lines.

4.5 Materials and Methods

4.5.1 Gene cloning and protein expression

The *OsCDPK5* (AF194414) cDNA clone was isolated from rice cDNA library as a positive clone by the solid phase phosphorylation screen with OsMPK5. The positive clones carrying *OsCDPK5* cDNA were excised in vivo from the λZAP Expression vector with the aid of helper phage M13 (Stratagene, La Jolla, CA). The full-length *OsCDPK5* cDNA was sequenced and subcloned into the GST expression vector pGEX-5x-3 with EcoR I and Xho I restriction sites. Both recombinant proteins His-OsMPK5 and GST-OsCDPK5 were expressed and purified from *E.coli* BL21 respectively with the nickel sepharose beads (Nickle sepharous) and glutathione sepharose beads according to manufacturer's instruction (Pierce).

4.5.2 Protein binding and kinase assays *in vitro*

In vitro protein binding and kinase assays were performed with the same procedure as mentioned previously in Chapter 2 and Chapter 3. Subcloning was made in the expression vector pET28c to divide the full-length OsMPK5 into two gene fragments encoding the C-terminal 22 kD and N-terminal 20 kD peptides. After purifying the recombinant proteins (two truncated versions of OsMPK5, His-OsMPK5 and GST-OsCDPK5), 1ug of each protein was used to perform protein binding and kinase assay *in vitro*. The polyclonal antibody against C-terminal region of OsMPK5 and the monoclonal antibody against GST were used respectively to detect His-OsMPK5 and GST-OsCDPK5.

4.5.3 Co-immunoprecipitation

Total proteins were extracted from leaves of two-week-old rice plants by grinding in liquid nitrogen and homogenized in the lysate buffer (20mM Tris-Hcl pH7.9, 150mMNacl, 1% Triton X-100, 5mM EDTA, 1mM phenylmethylsulfonyl fluoride). For immunoprecipitation, disuccinimidyl suberate (DSS) was used to ensure that the antibody is chemically cross-linked to the protein G during the antigen binding, wash and elution steps. Briefly, protein G sepharose (GE Healthcare) was washed 3 times with the washing buffer (20mM HEPES, pH 7.4, 1xPBS, 0.2mM EDTA, 5% glycerol) and was mixed with 5ul anti-OsMPK5 antibody. After incubation for 2 hours at 4 °C, the mixture was washed once with the washing buffer, added 2mM DSS to incubate for 30 minutes at room temperature, and washed twice with the buffer (20mM Tris-HCl, pH8.0, 150mM NaCl). The protein G conjugated with OsMPK5 antibody was mixed with the whole protein from leaf tissue of two-week-old rice plants followed by at least 4 hours incubation at 4 °C. After 4 times wash with the washing buffer and addition of protein loading buffer, the co-immunoprecipitated complexes were separated by 10% SDS-PAGE. Western blot analyses were used to detect OsMPK5 and OsCDPK5 proteins respectively with the polyclonal antibodies (against the C-terminal region of OsMPK5 and the N-terminal region of OsCDPK5, respectively).

4.5.4 RT-PCR and RNA blot analyses

Total RNAs were isolated from leaf tissues of two-week-old rice plants using the Trizol reagent (Invitrogen). For RNA blot analysis, 20ug total RNAs were separated with 1.2% agarose gel and transferred to nylon membranes. The hybridization was performed

overnight at 60 C in the PerfectHyb solution (Sigma) using [α-^{32}P] CTP labeled gene-specific probe (a 680 base pairs DNA sequence was generated from 3' region of *OsCDPK*5 by the digestion with restriction enzymes, Sal I and BamH I)). RNA blots were washed twice in 2x SSC for 15 min at 60 ºC, then twice in 1x SSC, 0.5% SDS for 20 min at 60 ºC, and finally in 0.1x SSC at room temperature for 10 min. For RT-PCR, reverse transcriptions were carried out using the SuperScript III RT kit according to the manufacturer's instructions (Invitrogen). PCR amplifications were performed using GoTaq GreenMaster mix (Promega) for 32 cycles with the following parameters: 95 ºC for 30 s, 59 ºC for 30 s, and 72 ºC for 1 min.

4.5.5 Chemical and wounding treatments

For chemical treatments, leaves of two-week-old rice plants were sprayed with ACC (0.1 mM) or ABA (0.1 mM) solution. Mechanical wounding was achieved by crushing rice leaves with a hemostat. Leaf tissues were collected at different time points and immediately frozen with liquid nitrogen.

4.5.6 Generation of transgenic *OsCDPK5* RNAi lines

Two RNAi constructs were made using the pANDA vector (Miki and Shimamoto, 2004) for silencing the single (the *OsCDPK5* RNAi-construct) or double (the *OsCDPK1+5* RNAi construct) genes. Specifically, cDNA fragments with 393 (OsCDPK5) or 435 (OsCDPK1) bp were amplified respectively from the 3' regions of both cDNAs using two pairs of primers (OsCDPK5 forward 5' CAC CGA TTG TTG AGG CAA TTG ACA G 3', OsCDPK5 reverse 5' GGA TCC AAT GAC TGC CCT

TTG TTC AG 3', OsCDPK1 forward 5' GGA TCC AAG CAA TCG ACA GCA ACA C

3', and OsCDPK1 reverse 5' ACT CAA GAC ACC CTC AAC ATC 3'). These

fragments were cloned into the pANDA RNAi vector using the Gateway cloning

approach (Invitrogen). Subsequently, the RNAi constructs were verified by DNA

sequencing and introduced into *Agrobacterium tumefaciens* strain EHA105 by

electroporation. Agrobacterial strains carrying the dsRNAi constructs were grown

overnight in AB induction medium (Winans et al., 1988) containing 50 ug/mL

hygromycin and 100 uM acetosyringone. Bacterial cells were collected by centrifugation

and resuspended in induction medium to OD600 of 0.1 for rice transformation. The

Agrobacterium-mediated transformation was performed using vigorously growing calli

derived from mature embryos of rice cultivar Kitaake according to the method described

previously (Hiei et al, 1994).

```
OsCDPK5    MGLCSSSSARRDAGTPGGGNGAGNKDNAGRKGIVACG---------KRTDFGYDKDFEAR
OsCDPK1    MGACFSSHTATAAAD--GGSGKRQQRKGDHKGKLPDGGGGEKEKEAARVEFGYERDFEGR
           ** * ** :    *.   **.*  :: :..:** :. *        *.:***::***.*

OsCDPK5    YALGKLLGHGQFGYTFAAVDRRSSERVAVKRIDKNKMVLPVAVEDVKREVKILKALQGHE
OsCDPK1    YQVGRLLGHGQFGYTFAATDRASGDRVAVKRIDKAKMVRPVAVEDVKREVKILKELKGHE
           * :*:************** .** *.:******** *** ************** *:***

OsCDPK5    NVVHFYNAFEDDNYVYIVMELCEGGELLDRILAKKDSRYSEKDAAVVVRQMLKVAAECHL
OsCDPK1    NIVHFYNAFEDDSYVYIVMELCEGGELLDRILAKKNSRYSEKDAAVVVRQMLKVAAECHL
           *:**********.**********************:*********************

OsCDPK5    HGLVHRDMKPENFLFKSTKEDSSLKATDFGLSDFIRPGKHFRDIVGSAYYVAPEVLKRKS
OsCDPK1    HGLVHRDMKPENFLFKSTKEDSPLKATDFGLSDFIKPGKKFHDIVGSAYYVAPEVLKRRS
           ********************* .************:***:*:*****************:*

OsCDPK5    GPESDVWSIGVITYILLCGRRPFWDKTEDGIFKEVLKNKPDFRRKPWPNITPCAKDFVQK
OsCDPK1    GPESDVWSIGVITYILLCGRRPFWNKTEDGIFREVLRNKPDFRKKPWPGISSGAKDFVKK
           ************************:*******:***:******:****.*:. ******:*

OsCDPK5    LLVKDPRARLTAAQALSHEWVREGGQASDIPLDISVLHNMRQFVKYSRFKQFALRALAST
OsCDPK1    LLVKNPRARLTAAQALSHPWVREGGEASEIPVDISVLSNMRQFVKYSRFKQFALRALAST
           ****:************* ******:**:**:***** ***************************

OsCDPK5    LNAEELSDLRDQFNAIDVDKNGTISLEELKQALAKDVPWRLKGPRVLEIVEAIDSNTDGL
OsCDPK1    LKEEELADLKDQFDAIDVDKSGSISIEEMRHALAKDLPWRLKGPRVLEIIQAIDSNTDGL
           *: ***:**:***:******.*:**:**::: *****:***********::********

OsCDPK5    VDFEEFVAATLHVHQLVEHDTEKWKSLSQAAFDKFDVDGDGYITSDELRMQ--TGLKGSI
OsCDPK1    VDFEEFVAATLHIHQMAELDSERWGLRCQAAFSKFDLDGDGYITPDELRMVQHTGLKGSI
           ************:**:.* *:*:*   .****.***:*******.***** ** *******

OsCDPK5    DPLLEEADIDRDGKISLDEFRRLLKTASMSSRNVQTPRSVHRS---
OsCDPK1    EPLLEEADIDKDGRISLSEFRKLLRTASMS--NLPSPRGPPNPQPL
           :*********:**:***.***:**:***  *: :**.  ..
```

Figure 4.1 Alignment of protein sequences between OsCDPK5 and OsCDPK1.

OsCDPK5 (NP-001059444.1) and OsCDPK1 (NP-001045751) fall into the same group of the rice CDPK family. They share highly conserved kinase domain and EF hand regions. Red color indicates kinase domain. Green color indicates EF hand domains.
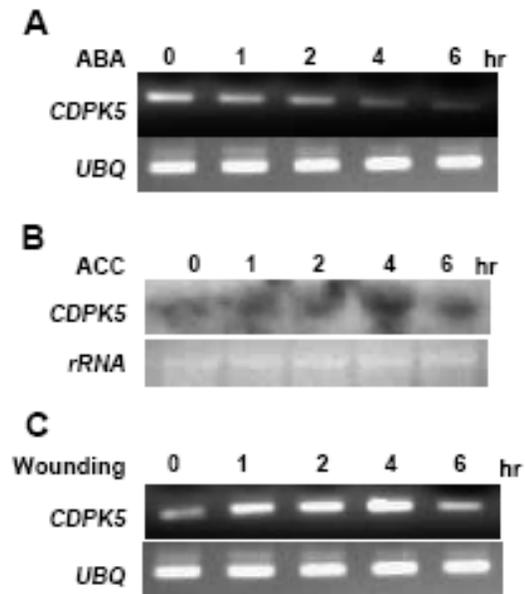
Figure 4.2 Expression of OsCDPK5 in response to ABA, ACC and wounding.

The mRNA levels of *OsCDPK5* were determined after treated with 0.1 mM ABA (A, detected by RT-PCR), 0.1 mM ACC (B, detected by RNA-gel blot) and wounding (C, detected by RT-PCR).

A

Ca2+ (mM)   0.01   0.1   1.0   10   10+4mMEGTA

Kinase Assay   P-HIS-CDPK5

                                                P-HIS-MPK5

Loading   HIS-CDPK5

                                                HIS-MPK5

B

Ca²⁺   0   2   4   8   16   32   64   mM

                                      P-GST-OsCDPK5

Ca²⁺   0   2   4   8   16   mM

                                P-GST-OsCDPK5

                                P-His-OsMPK5
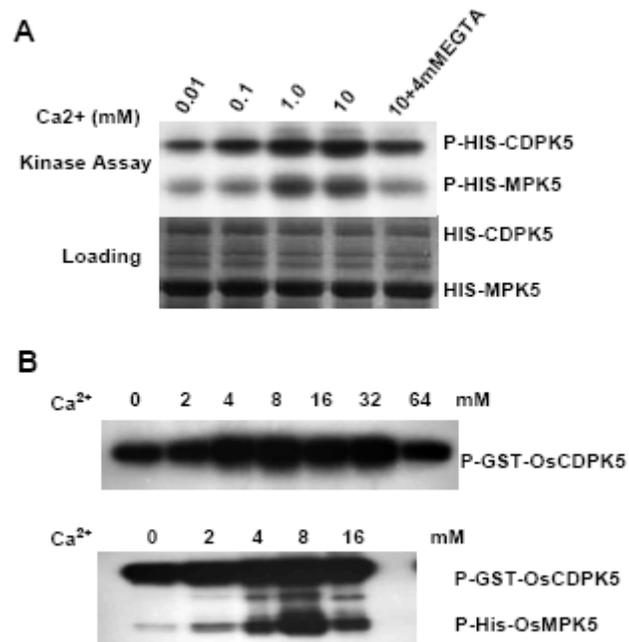
Figure 4.3 Calcium concentration regulates the OsCDPK5 kinase activity.

A, Calcium (10-fold increase) up-regulated OsCDPK5 kinase activity and increased the phosphorylation of OsMPK5. B, Calcium (2-fold increase) up-regulated OsCDPK5 kinase activity and increased the phosphorylation of OsMPK5.

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Figure 4.4 OsCDPK5 phosphorylates OsMPK5 C-terminal region.

OsCDPK5 was mixed with OsMPK5-C or OsMPK5-N in the kinase buffer supplemented with γ-32P-ATP. ^{32}P incorporation was measured by X-ray film, and the equal protein loading was estimated based on Coomasie-blue staining.

Figure 4.5 Interaction of OsCDPK5 with OsMPK5 *in vitro* and in vivo.

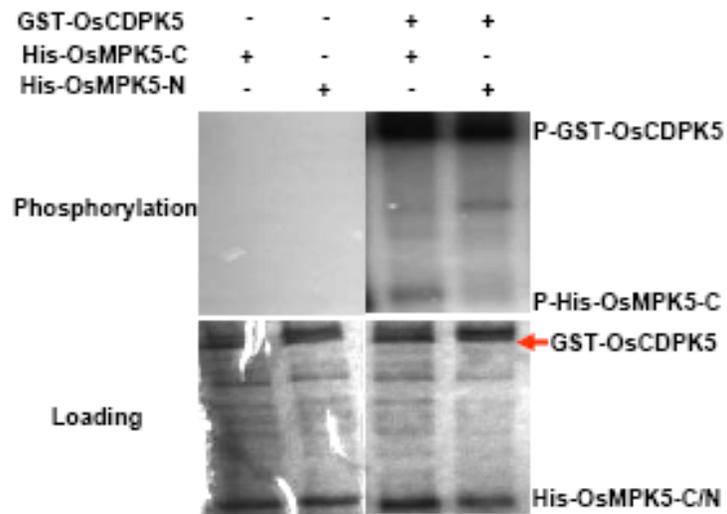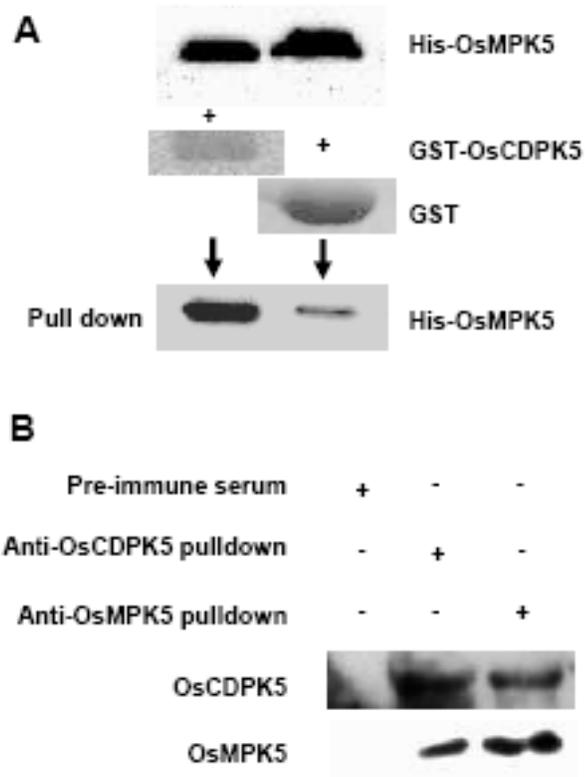A. *In vitro* binding between OsCDPK5 and OsMPK5. The protein binding assay was conducted by mixing His-OsMPK5 and GST-OsEIL1 in the binding buffer followed by incubation for 2 hours at 4 ℃. The glutathione-agarose beads were used to pull down GST or GST-tagged proteins. After the 10% SDS-page gel separation, a polyclonal antibody against OsMPK5 was used to detect His-OsMPK5, the GST monoclonal antibody was used to detect GST and GST-OsEIL1. B. In vivo interaction between OsCDPK5 and OsMPK5 demonstrated by the coimmunoprecipitation. The whole proteins were isolated from leaf tissues of two-week-old rice plants. The polyclonal antibodies against OsCDPK5 and OsMPK5 were used respectively to bind their own antigens. Protein G was used to pull down the affiliated antigen and antibody complexes. After incubation for 4 hours at 4 ℃, the protein complex was separated by 10% SDS-page gel. Western blot was used to detect OsCDPK5 and OsMPK5.
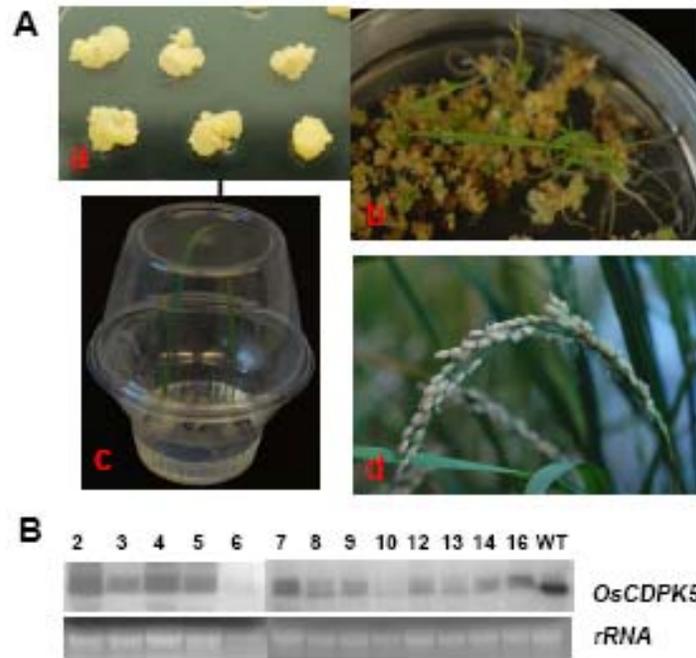
Figure 4.6 Generation and confirmation of *OsCDPK5* transgenic RNAi lines.

A, Generation of the transgenic RNAi lines. The process involves the construction of
OsCDPK5 RNAi plasmid, the transformation of the construct into *Agrobacterium*, the
co-cultivation of *Agrobacterium* with rice calli (a), the selection of transgenic calli, and
regeneration of transgenic shoots (b) and plantlets (c). Transgenic rice plants were grown
in a greenhouse for bioassays and seed production (d). B, Molecular confirmation of
OsCDPK5 RNAi lines. Total RNAs were isolated from leaf tissues of T0 transgenic
plants (*OsCDPK5* RNAi-lines #2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 13, 14, 16, wild type). A total
of 20ug RNAs from each line were loaded in agarose gel. OsCDPK5 mRNA level was
detected by RNA blot analysis.
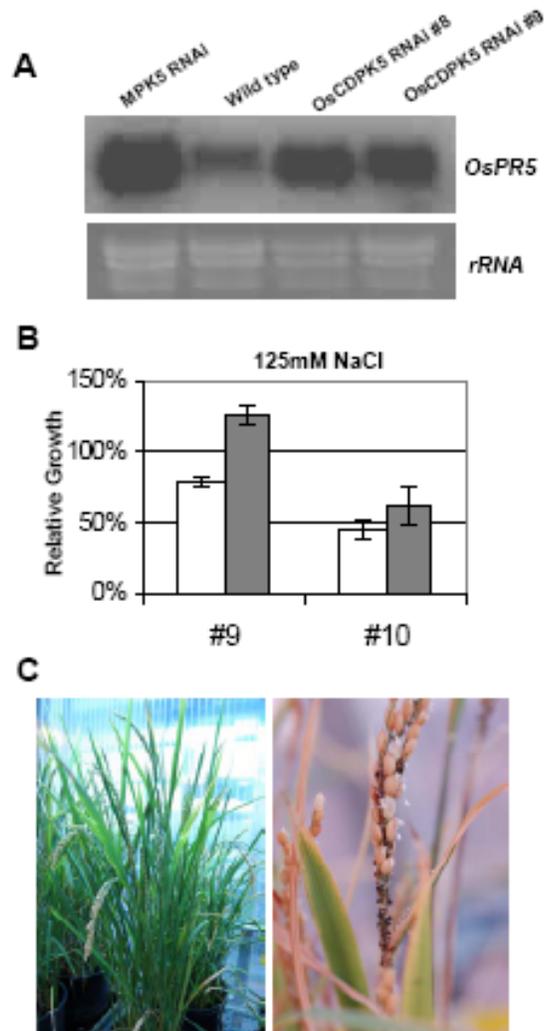
Figure 4.7 Effects of OsCDPK5 on defense gene expression and stress tolerance

A, Negative regulation of *OsPR5* expression by *OsCDPK5*. Total RNAs were isolated from leaf tissues of two-week-old rice lines and analyzed by RNA blot analysis. B. *OsCDPK5* RNAi lines exhibited increased salt tolerance. White bars indicate wild types. Grey bars indicate *OsCDPK5* RNAi lines. Standard deviation is indicated by error bars. C. *OsCDPK5* RNAi lines appeared to be more susceptible to aphid infestation.

Chapter 5 Conclusions and Future Directions

The OsMPK5-mediated stress signal transduction in rice was explored here by identifying and characterizing its substrates using molecular, biochemical and genetic approaches. Biochemical screening has been broadly used in yeast and animal systems to identify the interacting proteins based on the structural and functional stability of proteins *in vitro*. In this study, fourteen putative substrates of OsMPK5 were isolated from the rice cDNA expression library using an in-situ solid phase phosphorylation screening. Most of these substrates contain at least one potential MAPK phosphorylation site, and several of them are implicated to be associated with biotic and abiotic stress responses. Two of the substrates have been further confirmed by the kinase assay as the phosphorylation substrates of OsMPK5 *in vitro*, indicating that the *in-situ* solid phase phosphorylation screening is a feasible method for identifying plant kinase substrates. Although phosphorylation screening based on the *in vitro* kinase assay was shown to successfully isolate the putative substrates, verification of phosphorylation events in vivo is necessary to further confirm that these are indeed the substrates of OsMPK5. In addition, the combination of chemical and genetic strategy may be an alternative for screening the phosphorylation substrates of kinase *in vivo* (Dephoure et al, 2005). This strategy is based on the *in vivo* phosphorylation and *in vitro* screening, where kinase is mutated by genetic method and specifically binds to the modified form of ATP. The two-dimension gel electrophoresis and phosphorylation staining are used to isolate the positive phosphorylation based on the differentiation of phosphorylation staining between wild type and the mutant.

Based on the diverse substrates identified by the phosphorylation screening, OsMPK5 is likely involved in multiple stress signaling pathways. Our biochemical and genetic analyses reveal that OsMPK5 regulates OsEIL1 protein stability and ethylene responsive gene expression, suggesting that OsMPK5 is involved in regulation of ethylene signaling pathway. Genetic and biochemical studies in *Arabidopsis* have shown that AtMPK3/6 is genetically located between CTR1 and EIN3 and capable of directly phosphorylating EIN3. However, the role of MPK in ethylene signaling pathway has not been clearly demonstrated. The future experiments may focus on the identification of MAPKK or MAPKKK components in ethylene signaling pathway. Biochemical approaches (interaction screening and phosphorylation screening) can be used to further identify their up-stream MAPKKs interacting with OsMPK5 or AtMPK3/6 and then MAPKKKs. Their interaction with CTR1 or receptors in ethylene signaling pathway can be characterized. In addition, genetic analysis is necessary to characterize any change of these kinases' phosphorylation in response to ethylene or other stresses and their roles in the ethylene-induced triple phenotypes. Besides OsMPK5, other MAPKs may also modify components in ethylene signaling pathway via phosphorylation.

It has been proposed that the MAPK pathway may cross-talk with the CDPK pathway (Ludwig et al., 2005). However, the underlying mechanism is still unknown. We demonstrated here that OsCDPK5 interacts with and phosphorylate OsMPK5 *in vitro* and *in vivo*, and regulates the downstream ethylene-responsive *OsPR5* gene expression. To further elucidate the cross-talk mechanism among the CDPK, MAPK and ET signaling pathways, the following experiments can be performed. First, more careful analyses are needed to confirm OsCDPK5's involvement in ethylene signaling transduction, including

the analyses of the OsEIL1 protein level and other ethylene responsive gene (e.g., *OsERF1*) expression in OsCDPK5 RNAi lines. Second, calcium positively regulates OsCDPK5 autophosphorylation and its phosphorylation of OsMPK5 *in vitro*. However, the *in vivo* regulation of calcium on both kinase activities needs to be examined, which may regulate the ethylene responsive gene expression. Third, more experiments are needed to elucidate the effect of OsCDPK5-mediated OsMPK5 phosphorylation on OsEIL1 (OsEIL1 phosphorylation, protein stability and activity). If the three proteins (OsCDPK5, OsMPK5, and OsEIL1) form a protein complex, the future experiments may also include the demonstration that any interaction among two of these proteins may regulate ethylene signal transduction. In addition, we may study the phosphorylation events of both kinases in response to ABA or ethylene and determine the specificities of phosphorylation sites in response to different hormones.

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