이학박사 학위 논문

A Study on the Signaling Pathway of Salt- and *Bradyrhizobium japonicum*-Activated MAPKs in Soybean

콩에서 염과 *Bradyrhizobium japonicum* 에 의해 활성화되는 MAPKs 의 신호전달 과정에 관한 연구

2013년 2월

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ABSTRACT

Soybeans MAPKs were first reported in early 2000s. However, identification and signaling pathway of soybean MAPKs was barely known. Therefore, I studied the signaling pathway and regulation mechanism of a salt-stress activated 47-kD MAPK, GMK1, and *Bradyrhizobium japonicum* culture filtrate activated two MAPKs, GMK1 and GMK2. Concerning the salt stress, activity of GMK1 increased with increasing salt concentrations up to 300 mM NaCl after 5 min of the treatment and was regulated by post-translational modification. I found that mastoparan, a heteromeric G-protein activator, also activated GMK1, and that *n*-butanol, a phospholipase D inhibitor, and neomycin, a phospholipase C inhibitor, inhibited its activity. Moreover, GMK1 activity was reduced by suramin, a heteromeric G-protein inhibitor, and by two inhibitors of phosphatidic acid (PA) generation after 5 min of 300 mM NaCl treatment. Endogenous PA levels were highest 5 min after induction of salt stress, and exogenous PA directly activated GMK1. These results suggest salt signaling passed from heteromeric G-protein to GMK1 via PA in early time of the stress. On the other hand, H_2O_2 also activated GMK1 even in the presence of PA generation inhibitors, but GMK1 activity was greatly decreased in the presence of diphenyleneiodonium, an inhibitor of NADPH-oxidase after 5 min of 300 mM NaCl treatment. On the contrary, the *n*-butanol and neomycin reduced GMK1 activity within 5 min of the treatment. Thus, GMK1 activity may be sustained by H_2O_2 10 min after the treatment. In the relationship between GMK1 and ROS generation, ROS generation was reduced by SB202190, a MAPK inhibitor in NaCl treatment. Moreover, ROS was increased in protoplast only overexpressing TESD-GMKK1, a GMK1 activator *in vitro,* but these effects were occurred at prolonged time of NaCl treatment. These data suggest that GMK1 indirectly regulates ROS generation. Further, GMK1 was translocated into the nucleus 60 min after NaCl treatment. The translocation is reduced by SB202190. Hydrogen peroxide induced translocation of GMK1 to nucleus and DPI disturbed

nuclear translocation of GMK1 in salt stress. It is means that translocation of GMK1 by 300 mM NaCl treatment is mediated by H_2O_2 in soybean.

Concerning the interaction between soybean and *Bradyrhizobium japonicum*, GMK1 and GMK2 were differently regulated by GCF treatment until 15 h of the treatment; GMK1 was continually activated, but activity of GMK2 was reduced after 3 h of the treatment. Moreover, GMK1 is regulated at post-translational level, but GMK2 was regulated at translational level. In the treatment of PA generation inhibitors, activities both GMK1 and GMK2 were reduced. However, in calcium signaling blocking, only activity of GMK2 was reduced. Therefore, these two MAPKs may play different roles in symbiotic interaction. However, the evidence of these MAPKs involvement on Nod factor signaling is not clear. Therefore, role of MAPKs in symbiotic interaction should be more elucidated.

Consequently, GMK1 is dually activated by PA and H_2O_2, and translocated to nucleus mediated by H_2O_2 in salt stress. Moreover, GCF treatment activated both GMK1 and GMK2, but these MAPKs were differently regulated in many aspects.

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ABBREBIATIONS

ACS	ACC synthase
BHT	butylated hydroxytoluene
cDNA	complementary DNA
CM-H_2DCFDA	5-(and-6)-chloromethyl-2,7′-dichloro dihydrofluorescein diacetate, acetyl ester
CWP	cell wall protein
DPI	diphenyleneiodonium
DTT	dithiothreitol
DNA	deoxyribonucleic acid
EDTA	ethylene diamine tetra acetic acid
EST	expressed sequence tag
FITC	fluorescein isothiocyanate
GCF	genistein induced *B. japonicum* culture filtrate
GFP	green fluorescent protein
IPA	isopropanol
kD	kilodalton
MBP	myelin basic protein
NADPH	nicotinamide adenine dinucleotide phosphate
PA	phosphatidic acid
PCR	polymerase chain reaction
RNA	ribonucleic acid
RT	reverse transcription
SDS	sodium dodecyl sulfate

SOD	superoxide dismutase
TCA	trichloroacetic acid
TLC	thin-layer chromatography
XTT	2,3-bis-(2-methoxy-4-nitro-5 -sulfophenyl)-2H-tetrazolium-5-carboxanilide
YEM	yeast mannitol broth

Chapter 1. General introduction

Plants cannot escape from harmful stresses including low temperature, wound, salt, hyper-osmotic and hypo-osmotic conditions because of their sessile life style. To protect themselves from the stresses, plants transfer the signal to inner cell area and produce many types of secondary metabolites for defense and regulation of development. In this context, accurate signal transduction is very important to plants for survival in various stresses. However, signaling pathway of various abiotic stresses is not well-understood in plants, especially soybean MAPK (mitogen-activated protein kinase) signaling pathway in salt stress. Moreover, role of MAPKs in symbiotic interaction is also hardly elucidated. Therefore, more elaborated MAPK signaling studies are needed.

1. Mitogen-activated protein kinase

MAPK, one of well-characterized signaling pathway modulators, is related with various biotic, abiotic stresses, symbiotic interaction and hormones in eukaryotes. MAPK is activated by upstream kinase, MAPK kinase (MAPKK), which is also activated by MAPKK kinase (MAPKKK). This modulation is also well-conserved in eukaryotes.

MAPK is activated by the dual phospholyation of threonine and tyrosine residues of the TXY motif that is located between subdomain 7 and 8 of the kinase catalytic domain. X can be Glu (E), Pro (P), or Gly (G). MAPK consists of 3 subfamily, based on the phosphorylation motif. Extracellular signal-regulated protein kinses (ERKs) have TEY motif, whereas Jun kinases have TPY and TGY motif in p38 MAPKs. These kinases are activated by different stress signals in mammalian cells (Kyriakis and Avruch, 1996). However, plants MAPKs have only a TEY motif. Therefore, plants have different regulation mechanisms on MAPKs compared with mammalian MAPKs (Ashraf, 1994).

1-1. MAPK signaling pathway by biotic stress and hormone

When pathogens attack, plants trigger Pathogen-Associated Molecular Pattern (PAMP). PAMP is associated with immune response as well as MAPK activation.

Flagellin of *Pseudomonas syringae* (flg22) is detected by the FLS2/BAK1 receptor. The detection leads to activation of MAPK cascade, AtMEKK1/AtMKK1 and AtMKK2/ AtMPK4. This signaling blocks action of WRKY33, but activates WRKY53. Therefore, MAPK signaling is related to WRKY genes expression when plants meet pathogen (Miao et al., 2007; Pitzschke et al., 2009; Pitzschke et al., 2009).

One of well-characterized plants MAPK signaling pathways is an ethylene signaling. Ethylene is generated by various stresses and signals such as cold, salt, pathogen and pathogen elicitors. These stresses activate AtMPK6. Activated AtMPK6 positively regulates ACS on cytoplasm (Liu and Zhang, 2004). Therefore, ethylene production is increased by the stresses, and the increase is detected by an ethylene receptor, ETR/ERS. These receptors positively regulate CTR, while the MAPKKK negatively regulates downstream MAPKK. Ethylene inactivates ETR receptor and leads to CTR inactivation (Guo and Ecker, 2004). CTR inactivation is a cause of AtMKK9 and AtMPK3/6 activations. Activated AtMPK3/6 as well as EIN2 phosphorylate EIN3 and trigger expression of ethylene response gene. This signaling is depicted in Figure 1-1.

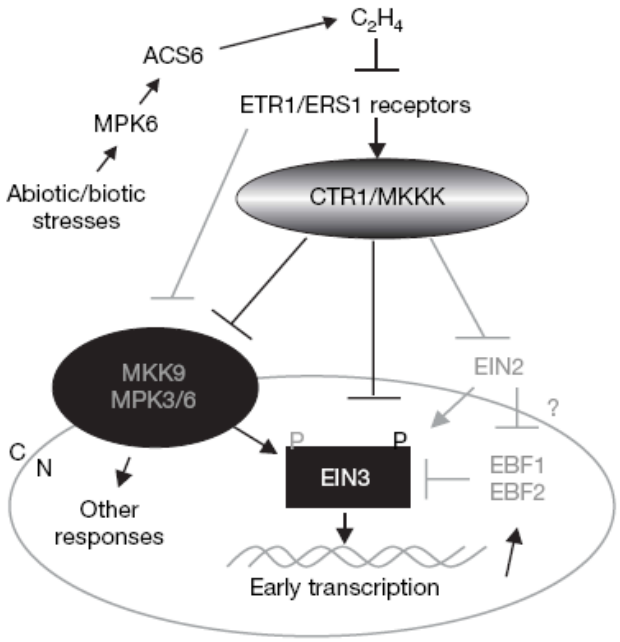


Figure 1-1. Model of the bifurcate MAPK cascades in ethylene signaling (Yoo et al., 2008).

1-2. MAPK signaling pathway in abiotic stress

MAPK is activated by various abiotic stresses including cold, wounding, osmotic and salt stress. It is difficult to study MAPK signaling pathway in abiotic stress because all of these stresses induce ROS and calcium influx (Rizhsky et al., 2004). Therefore, upstream of MAPK cascade is not well-characterized in abiotic stress. However, stress-specific expressions of MAPK genes are increased or the activities are increased when plants is stressed.

1-2.1. Cold stress

Most of plants live in climate changing area. High and low temperatures affect their survival and regulation of development. In this case, MAPK activation is also involved in the environmental changes. AtMPK6 and AtMPK4 are activated by

cold (Ichimura et al., 2000) and upstream MAPKKK, AtMEKK1 is increased its transcription level by the stress (Mizoguchi et al., 1996). AtMEK2 is also up regulated by cold stress and the kinase interacts with AtMPK6 and AtMPK4 in yeast two hybrid (Mizoguchi et al., 2000). AtMEKK1 is a functional upstream of AtMEK1 and AtMEK2. Thereore, MAPK module AtMEKK1-AtMEK2-AtMPK4/6 is confirmed in cold stress.

1-2.2. Oxidative stress

Most abiotic stresses, such as touch, cold, salinity, UV irradiation and ozone disrupt the metabolic homeostasis of plant cells, and generate oxidative stress (Mittler et al., 2002). Moreover, pathogen attack and hormone also generate the stress. Reactive oxygen species (ROS), is a mediator of oxidative stress, leads to damage to molecules with consequential injury to cells or tissue (Gill and Tuteja., 2010). Therefore, removal or neutralization of the molecule is very important to plants. Plants used endogenous antioxidants (e.g., catalase, glutathione, superoxide dismutase) and exogenous antioxidants (e.g., vitamins A, C, E, bioflavonoids, carotenoids) to reduce the stress molecules.

Plants overcome oxidative stress with scavenger enzymes such as catalases, which decomposing H_2O_2, and MAPK with regulation of the enzyme. For example, *Arabidopsis* CAT1 is regulated by ABA, and MAPKK inhibitor, PD98059, reduced ABA-mediated CAT1 expression (Xing et al., 2007). In addition, the *Arabidopsis mkk1* and *mpk6* mutants were altered in their responses to ABA and desiccation stress. These results suggested that AtMKK1-AtMPK6 regulates H_2O_2 metabolism through CAT1 (Xing et al., 2008).

The AtMEKK1-AtMPK4 cascade also has an important role in ROS metabolism and MEKK1may be specifically required for the activation of MPK4 by H_2O_2 (Nakagami et al., 2006). Moreover, H_2O_2 was accumulated in *mpk4* mutant, suggesting that MPK4 may induce or stabilize CAT2 activity (Cristima et al., 2010). In addition, ANP1, *Arabidopsis* MAPKKK, is activated by H_2O_2. This kinase

activated downstream MAPKs of AtMPK3 and AtMPK6 in the stress (Kovtun et al., 2000). These data suggest that MAPK cascades are not only activated by ROS but also regulate ROS generations.

1-2.3. Wounding

Many physical injuries caused by herbivore or insect attack result in wound stress. Plants express several defense-related genes in the stress (Lawton and Lamb, 1987; Brederode et al., 1991). The expressions of these genes are activated through signaling pathways that include various protein kinases.

The first study of the activation of MAPK in wounding was reported in tobacco (Seo et al., 1995) and the MAPK is an AtMPK3 orthologus, WIPK (wound induced MAPK). AtMPK4 and AtMPK6 are rapidly activated by wounding in *Arabidopsis* (Ichimura et al., 1998). MMK4 was also activated in wounded alfalfa leaves (Borgre et al., 1997). Molecular characterization of *StMPK1* revealed its transcriptional up-regulation upon wounding in potato (Blanco et al., 2006). In soybean, 49 kD MAPK is also activated by wound (Lee et al., 2010). However, signaling pathway or MAPK module is not well characterized in the stress.

2. Salt stress

Salt is a major environmental stress, affects 19.5% of irrigated land (Neto et al., 2006) and most crops are sensitive to salt. Salt stress is occurred by limited rainfall, poor water supplement, high temperature and evaporation. High concentration of salt reduces development and productivity to plants. Even though soybean is a moderate salt-tolerance plant, soybean products are reduced in 5 dS/m salt (Ashraf, 1994). Salt stress also affects to germination. Soybean seeds were reduced its germination in 0.05% NaCl (Phang et al., 2008) and *Arabidopsis* seeds also disturbed in 20 mM NaCl (Borsani et al., 2001).

Sodium accumulation induces nutrient deficiency, osmotic stress and ionic toxicity. High concentration of sodium disturbs inorganic nutrient uptake such

as calcium, zinc, iron, phosphate and potassium. Cell is escaped from inner cell area to outer membrane and plasma membrane is being separated from cell wall. This phenomenon is called plasmolysis. High concentration of salt lead to osmotic stress and continuation of the stress makes a cell death (Munns et al., 2002). Moreover, most of potassium ion is replaced by sodium ion. Potassium acts as a cofactor of some enzyme, but sodium cannot replace the role of potassium. Therefore, high concentration of sodium ion could be toxic to plant (Chinnusamy et al., 2006).

Arabidopsis and soybean are glycophyte that is easily damaged by saline and inhibited growth and development in the high concentrations of NaCl. The glycophytes could be damaged at all developmental stages. For example, embryos treatment with 150 mM NaCl show callus accumulation, abnormal embryo formation, and cell death in *Arabidopsis*. Their seed germination and seedling stages are the most sensitive. Moreover, germination and subsequent plant growth are markedly impaired, and cell death is occurred even at 50 mM NaCl (Cheong and Yun, 2007).

2-1. Salt stress signaling and tolerance

High salinity also generates secondary damages to plant (Zhu, 2001). To survive from the stress, plant developed defense machinery such as SOS system, re-established ion homeostasis and regulation of osmolytes.

2-1.1. SOS system

Excretion of sodium ion is very important to cell during salt stress. Salt Overly Sensitive 1 (SOS1), Na^+/H^+ antiporter, is localized in plasma membrane and is essential for salt tolerance (Shi et al., 2002). SOS1 has 12 transmembrane domain and long tail in cytosol (Shabala et al., 2005). SOS1 effluxes sodium ion and is directly required for the uptake of potassium (Wu et al., 1996; Fraile-Escanciano et al., 2010). SOS5, contained AGP and fasciclin domain, is a thought to be candidate of sodium ion sensor, however the evidence is not sufficient (Mahajan et al., 2008).

SOS2 is a serine/threonine protein kinase and SOS3 is known as a CBL4, acts as a calcium sensor. SOS2 has FISL motif in C-terminal domain interacting with SOS3.

Salt stress induces ABA and increases Ca^{2+} level in cytosol. SOS2/SOS3 complex is activated by Ca^{2+}, and the complex regulates SOS1 activation. This complex also regulates V-ATPase and NHX1 in vacuole. Therefore, intracellular Na^{+} levels are decreased. This regulation mechanism is maintained by gene expression of SOS1 and SOS4. The expressions of genes are also regulated by SOS2/SOS3 complex. This regulation mechanism is depicted in Figure 1-2.

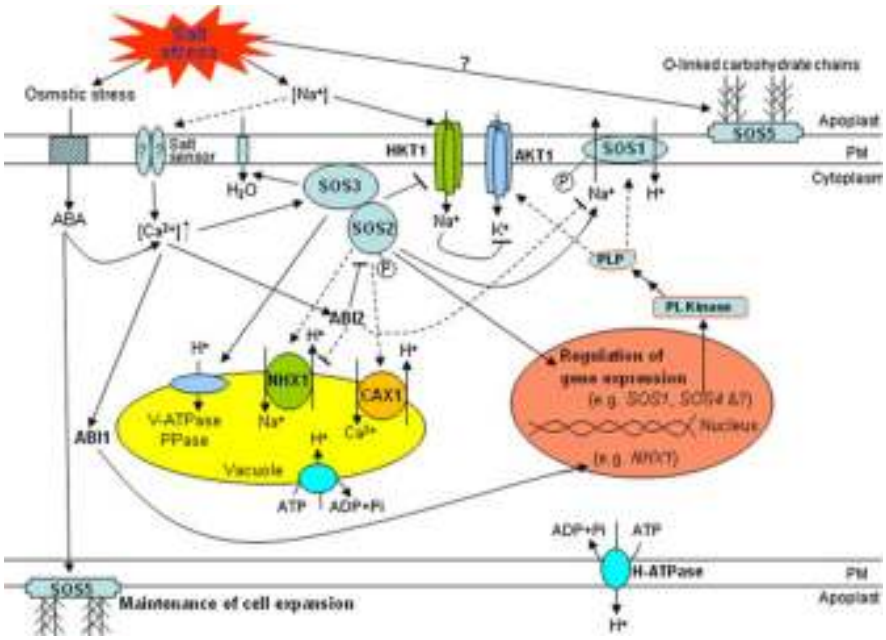


Figure 1-2. Regulation of ion homeostasis by SOS signaling pathway for salt stress adaptation (Turkan and Demiral, 2009)

2-1.2. Ion regulation in salt stress

Plants have various ion channels and transports. These enzymes regulate ion concentration in intracellular space. High concentration of NaCl in extracellular

space activates K^+-selective, Na^+-selective and non-selective channel and transporter in plasma membrane and vacuole (Amtmann and Sanders, 1999; Blumwald et al., 2000).

HKT1 is the first reported K^+ carrier protein, encoded as a K^+ high affinity domain. HKT1, is a Na^+-coupled K^+ transporter, contribute to reduction of permeability of Na^+ and salt stress tolerance (Rubio et al., 1996; Rubio et al., 1999). *Arabidopsis* homologus of HKT1, AtHKT1, determines salt-tolerance by controlling Na^+ entrance in root (Rus et al., 2001).

AKT1 plays a principle role in K^+ uptake in plant roots, however the protein has unlikely to play a role in inadvertent sodium uptake (Hirsch et al., 1998; Spalding et al., 1999). AKT1 is a member of the Shaker-like K^+ channel and additional members of this Shaker-like family, AKT2/AKT3, are weakly uptake K^+ to intracellular area and are expressed in both source and sink phloem tissues. Abscisic acid (ABA) up-regulates AKT2 expression, suggesting AKT2 participates in ion equilibrium during drought stress (Lacombe et al., 2000).

H^+-ATPases (V-ATPase) participate in salinity stress tolerance by Na^+ extrusion from the cytosol and compartmentation within various intracellular compartments (e.g., endoplasmic reticulum, Golgi, and vacuole), respectively (Sze et al., 1999; Morsomme and Boutry, 2000; Ratajczak, 2000). The other electrogenic proton pump, the H^+-translocating pyrophosphatase (PPiase), coexists with the V-ATPase in the vacuolar membrane and contributes to maintenance of vacuolar acidity under stress conditions (Maeshima, 2000). H^+ extrusion from the cell by P-ATPase or into the vacuolar lumen by H^+-ATPase or H^+-PPiase creates an electrochemical gradient. This proton force is used by secondary transporters, such as plasma membrane and tonoplast Na^+/H^+ antiporters, to couple the downhill movement of protons to the extrusion or sequestration (Blumwald et al., 2000).

2-1.3. Osmolytes biosynthesis

Salt stress generates low water potential. This phenomenon leads to water

loss to plant. To overcome the stress, plant synthesize many osmolytes including sucrose and fructose, sugar alcohols (glycerol and methylated inositols), complex sugars (trehalose, raffinose, and fructans), ions (K^+), and charged metabolites [glycine betaine, dimethyl sulfonium propionate (DMSP), proline, and ectoine] (Cheong and Yun, 2007).

Accumulation of osmolytes does not affect intracellular metabolism because most of the molecules are typically hydrophobic. They act on plasma membrane surface and protein-protein complex as a low molecular chaperon (Hasegawa et al., 2000).

In overexpression line of several osmolytes synthase are shown various stress tolerance. For example, glycine betanine improve salt and cold tolerance by protecting photosynthetic protein complex (Holmstrom et al., 2000). Mannitol protects cellular structure by reducing hydroxyl radical (Shen et al., 1997). Moreover, proline protects plasma membrane and proteins in various stresses (Khedr et al., 2003; Ashraf and Foolad, 2007).

3. Soybean

Soybean has been supplying natural protein for human and animal because it contains 40% of protein in dry soybeans. Soybean also has a lot of oil and flavonids. Therefore, it is considered as a useful crop for human. Soybean was used various processed food including soy milk, tofu, soy sauce and fermented bean paste. Oil also used as a vegetative oil or biodiesel.

United States and Brazil are most producers of soybean. These two countries produce 62% of soybean in the world, and Argentina (19%), China (6%) and India (4%) produce soybean.

The name of *Glycine* was firstly introduced by Carl Linnaeus in 1737. The Glycine genus is subdivided into *Glycine soja* and *Glycine max* L.

3-1. Soybean in salt stress

Soybean is a moderately salt-tolerant plant, however high salt imposes damage to the plant. Germination of soybean seeds is delayed by 0.05% NaCl condition and higher than 0.1% NaCl completely decreases the germination (Abel and MacKenzie, 1964).

The seedling of soybean is considered to be more sensitive to salt stress than the germination stage (Hosseini et al. 2002). The growth rate of seedlings at 220 mM NaCl declined to 5% when compared with normal condition seedlings, and stunted growth was observed at 300 mM NaCl (Phang et al., 2008).

The quality of seeds is also affected by salt stress. Salt reduced the protein contents in soybean seeds (Chang et al. 1994; Wan et al. 2002). However, the effect of salt on oil content of soybean seeds is still unclear (Chang et al. 1994; Wan et al. 2002).

3-2. Salt tolerance in soybean

3-2.1. Gene regulation in salt stress

Calcium is an important molecule in stress response. GmSCA1 is a salt stress responsible protein, is localized in plasma membrane (Chung et al., 2000).

SPK1 and SPK2 are activated by osmosensors. These proteins phosphorylated Ssh1p and reduced its binding affinity toward the plasma membrane, potentially redirecting its PtdIns 3-kinase and PtdIns 4-kinase-stimulating activities to a different subcellular location. The result leads to the alternation of phosphoinositide metabolism (Monks et al., 2001). Such alternation may affect the stress responses by affecting Ca^{2+} signature or acting on cellular transcription events.

Calmodulin is a non-enzymatic protein, but it has a crucial role in calcium signaling in eukaryotic cells. In soybean, five calmodulin genes, *SCaM-1* to *SCaM-5*, are reported (Park et al., 2004). SCaM-1 and SCaM-4 activate Ca^{2+}/calmoduin-dependent enzymes, but other proteins are still not shown the functions. The expression of *ScaM-4* is greatly increased by NaCl treatment.

GmSTY1, encoding a putative dual-specificity protein kinase, is isolated

from the cDNA of GAL4 activation domain fusion library of soybean (Xu et al., 2006). The predicted polypeptide shares common characteristics with both the serine/threonine and tyrosine protein kinases. The expression of *GmSTY1* is induced by drought and salt stress, however not induced by ABA treatment, suggesting that it may belong to an ABA-independent pathway.

DREB proteins are important to mediate ABA independent pathways in salt stress signaling. They are transcription factors involved in various biotic and abiotic stresses in plants (Agarwal et al., 2006). At least 10 members of the DREB gene family are present in the soybean genome (Phang et al., 2008). Differential gene expression is observed in the DREB family. For example, the expression level of *GmDREB1* was rapidly induced in the salt-tolerant wild soybean under dehydration conditions, which was significantly increased than that in the salt-sensitive cultivar (Chen et al., 2006). GmDREB1 was shown salt tolerance in transgenic line. *GmRDEB1* overly expressed transgenic alfalfa was showed salt tolerance (Jin et al., 2010). G*mDREBa* and *GmDREBb* were also induced by salt, drought, and cold stresses in the leaves of soybean. However, *GmDREBc* was not significantly induced in leaves. It was induced in roots by salt, drought and ABA (Li et al., 2005).

3-2.2. Structural change in salt stress

Plant cell walls have complex structure and are regulated various components. They are composed of polysaccharides and proteins. The shape of cell wall is determined by auxin, BR and water potential, which is affect to plant growth. The component and composition of cell wall are different by its cell type.

Structural cell wall proteins (CWPs) comprise only 10% of the dry weight of plant cell walls, but they have important role in the extracellular matrix.

Proline is one of the components in the cell wall and proline-rich cell wall has sensitivity to external stimuli (Bernhardt and Tierney, 2000). Moreover, the proline-rich CWPs appear to covalently attach to cell wall components during

development during physical damage and/or elicitor treatment (Suzuki et al., 1993). *SbPRP3* encodes a putative proline-rich CWP induced by salicylic acid, virus infection and salt stress, and also regulated by circadian rhythm, ABA and jasmonic acid in soybean (He et al., 2002). Its function is still unclear, but it has possibility of concerning cell wall fortification during stress.

Plasma membrane stabilization is very important to tolerate salt stress. Plasma membrane is consisted with phospholipids, glycolipids, sterols and membrane protein. High concentration of salt is cause of membrane leakage of electrolytes and organic compound of cell (Shao et al., 1993). Phospholipid is degradated and lipid phase is changed during salt stress. When the soybean cultivar "Kaoshing" is subjected to salt, the content of phospholipid is reduced and the ratio of saturated to unsaturated fatty acids is altered (Huang 1996). Plasma membrane protein fraction and sterols of soybean root are not changed under salt stress, but unsaturated fatty acids and microsomal fraction are reduced 55% and 26%, respectively (Surjus and Durand, 1996). The elevated saturated fatty acid level could improve the density of plasma membrane, and this phenomenon leads to salt tolerance of soybean.

3-2.3. Soybean MAPKs

Soybean MAPKs are firstly reported at 2001. 49-kD MAPK is activated by wound and phosphatidic acid (Lee et al., 2001). However, the study could not suggest MAPK gene, only suggested SIMK orthologus is activated by the signaling cue. At the same year, pathogen elicitor-activated ~47 kD and ~44kD MAPKs (Tayler et al., 2001), and 46-kD MAPK rapidly activated by light (Yamagata et al., 2001) were reported. Elicitor-activated two MAPKs are activated by oxidative burst and regulated by various inhibitors. The ~44 kD MAPK is also regulated by calcium signal. However, these studies also could not suggested genes of the MAPKs.

A study of pathogen and salicylic acid activated 46 kD MAPK was reported. The kinase was immunoprecipitated by MMK1 antibody (Anstatt et al.,

2003).

Six soybean MAPK clones are firstly reported, and some of them are identified by studies of β-glucan elicitor (Daxberger et al., 2007) and *Bradyrhizobia* infiltration treatments (Lee et al., 2008). In addition, GmMPK4, a defense- and growth-related soybean MAPK (Liu et al., 2011) are reported. However, soybean MAPK signaling pathway and regulation mechanism is hardly elucidated yet. Moreover, soybean MAPK activation is not studied even in salt, cold, ozone as well as various hormones. Therefore, more elaborated MAPK studies are needed in soybean.

4. Nodulation

Many legume plants have symbiotic relationships with rhizobial bacteria to use nitrogen. Bacteria reduce nitrogen to ammonia which is a useful form for plants. This event is occurred in special organs, called "root nodules." The symbiotic interaction is initiated by plant flovonoids. Flavonoids induce synthesis of Nod factor and the factor is released into the rhizosphere, and it is recognized by plant roots. The perception of Nod factor in the plasma membrane induces a signaling pathway that uses calcium as a secondary messenger. Genetic analysis in legume species such as *Medicago truncatula* and *Lotus japonicus* are revealed many components which are essential for the Nod factor induced signaling pathway. Nod factor makes root hair curling in legume plant and bacteria entered root hair cell though the event, and the root makes symbiotic space, root nodule.

4-1. Nodulation signaling

Nod factor receptor was founded with mutant screening in *L. japonicus*, M. *truncatula*, and *P. sativum*. LjNFR1 and LjNFR5 were found in *L. japonicus* and thought to be essential for Nod factor perception (Madsen et al. 2003; Radutoiu et al. 2003). Both genes encode transmembrane receptor-like kinase with two to three extracellular lysine motifs (LysM) and an intracellular serine/threonine protein kinase domain, while LjNFR1 contains an apparently functional serine/threonine

kinase domain (Huse and Kuriyan 2002; Schenk and Snaar-Jagalska 1999).

Phospholipase C and Phospholipase D are considered as downstream effectors of Nod factor receptor. Both of phospholipase C and phospholipase D were found to be activated by Nod factor (den Hartog et al. 2001; den Hartog et al. 2003) and their requirement in signal transduction has been revealed by pharmacological studies. Root hair deformation and nodulin expression were inhibited by application of neomycin, an inhibitor of phospholipase C (Charron et al. 2004; den Hartog et al. 2001; Pingret et al. 1998). *n*-butanol and cyclopiazonic acid also inhibit nodulin expression and mastoparan-induced calcium spiking (Charron et al. 2004 ; Sun et al. 2007).

5. Objective

MAPK is activated by various biotic and abiotic stresses and its activity is dynamically changed in the stresses. Therefore, activation time point of MAPK is very important. However, many MAPK related studies did not considered this view point. Moreover, the regulation mechanism and signaling pathways of plant MAPKs under abiotic stresses are largely unknown.

Soybean MAPK is a firstly reported at 2000, but its function and signaling pathway is barely understood. Moreover, salt stress-activated soybean MAPK is not reported yet. Moreover, even though *Bradyrhzobium* culture filtrate-activated two MAPKs were also reported, the activated two MAPKs were not completely identified. Therefore, this study tried to identify salt stress-activated soybean MAPK in soybean and elucidate signaling pathway of the MAPK. Moreover, this study also challenged to identify *Bradyrizobium* culture filtrate-activated two MAPKs and regulation mechanism of the MAPKs on the filtrate treatment.

Chapter 2

Signaling Pathway of GMK1 in Salt Stress

Im JH, Lee H, Kim J, Kim HB, Kim S, Kim B, An CS (2012) Salt stress-activated mitogen activated protein kinase in soybean is regulated by phosphatidic acid in early time of the stress. J Plant Biol 55(4): 303-309

Im JH, Lee H, , Kim HB, Kim J, An CS (2012) GMK1 is dually regulated by phosphatidic acid and hydrogen peroxide and translocated to nucleus during salt stress. Mol. Cells (In press)

2-1. INTRODUCTION

Salt stress is one of the most important environmental factors limiting the productivity of crop plants. A high concentration of salt in the soil inhibits the growth and development of plants through osmotic stress and ionic toxicity (Munns and Tester, 2008). To survive from the stress, plant operates defense systems like detoxification, re-established homeostasis of ion and growth regulation (Zhu, 2001). Accordingly, plants have evolved complex mechanisms to perceive and respond to salt stress in order to survive.

2-1.1. Salt stress signaling pathway

Under salt stress, plants mostly activate three signaling pathways. First, Ca^{2+} activates calcium-dependent protein kinase (CDPK). In a maize protoplast transient expression system, the dominant negative form of CDPK blocked osmotic stress and the abscisic acid (ABA)-related pathway (Sheen, 1996). Second, the SOS (salt overly sensitive) pathway is activated by excessive Na^{+} (Zhu, 2000). Finally, many MAPKs are activated by hyperosmotic stress in plants (Munnik et al., 1999). Salt stress-induced MAPK (SIMK) is activated by 750 mM NaCl and a 38-kD MAPK is activated by the same salt concentration in alfalfa (Munnik et al., 1999). Two other MAPKs, wound-induced protein kinase (WIPK) in tobacco and stress-activated MAPK (SAMK) in alfalfa, are also activated by hyperosmotic stress, as well as by wounding and cold (Seo et al., 1995; Jonak et al., 1996). However, the upstream regulatory proteins in salt stress response are not well characterized.

2-1.2. Phosphatidic acid and hydrogen peroxide

Phosphatidic acid (PA) is a phospholipid that acts as a secondary messenger and transmits cell signaling downstream. Recently, many studies have suggested that PA activates MAPK in abiotic stress as well. PA generated from phospholipase Dα (PLDα) activates AtMPK6 in salt stress (Yu et al., 2010), and a 49-kD wound-activated MAPK is activated by PA in soybean (Lee et al., 2001) and

H_2O_2-induced MAPK activity is not detected in PLD mutants (Zhang et al., 2003). PA can be generated directly by phospholipase D (PLD) and indirectly by phospholipase C (PLC). These two phospholipases are regulated by heteromeric G-protein. Mastoparan stimulates the guanine nucleotide exchange reaction of the α subunit in animal heterotrimeric G-proteins. In plants, mastoparan activates PLC (Legendre et al., 1993), PLD (Dhonukshe et al., 2003), and nod factor signaling in *Medicago* (Pingret et al., 1998). Mastoparan also increases cellular Ca^{2+} levels (Ross and Higashijima, 1994), induces an oxidative burst, and activates AtMAPK6 in *Arabidopsis* (Miles et al., 2004).

H_2O_2 is another well-known MAPK activator that regulates various responses to biotic and abiotic stresses. For example, H_2O_2 directly activates the two *Arabidopsis* MAPKs through the action of oxidative signal-inducible 1 (OXI1) (Rentel et al., 2004); further, abscisic acid (ABA)-induced H_2O_2 activates MAPK in maize (Jiang et al., 2006). Moreover, H_2O_2 alters diverse cellular processes in a dose-dependent manner. High H_2O_2 concentration triggers cellular response of programmed cell death (Alvarez et al., 1998; Delledonne et al., 2001), whereas low H_2O_2 concentration blocks cell cycle progression and regulates plant development and stress response (Reichheld et al., 1999; Neill et al., 2002; Foyer and Noctor, 2005). Several hormones also increase the H_2O_2 levels, leading to enhanced stress tolerance (Dat et al., 1998; Yang et al., 2001; Xia et al., 2009).

Many stresses result in the generation of signaling molecules, such as PA and H_2O_2, and their direct involvement in MAPK activation has been well-established. However, their involvement in regulating MAPK activity is not understood.

2-1.3. Soybean MAPK

Soybean is an important crop for human diet, animal feed, and biodiesel, due to its high protein and oil content. Although it is known as a moderately salt-tolerant crop, its productivity is significantly hampered by salt stress. Therefore, a

better understanding of the salt stress responses and tolerance mechanisms in soybean is important for devising strategies to improve its productivity.

Recently, six soybean MAPK clones were reported, and some were identified by studies of β-glucan elicitor (Daxberger et al., 2007) and *Bradyrhizobium* infiltration treatments (Lee et al., 2008). In addition, a 48-kD MAPK was shown to be activated by wounding and salicylic acid (SA) (Lee et al., 2001; Anstatt and Tenhaken, 2003), and 47-kD and 44-kD MAPKs were found to be involved in oxidative burst signal transduction (Taylor et al., 2001). Moreover, a 46-kD MAPK was shown to be rapidly activated by light (Yamagata et al., 2001), and by GmMPK4, a defense- and growth-related soybean MAPK (Liu et al., 2011). Nevertheless, a salt stress-activated soybean MAPK has not been reported yet, and signaling pathways of soybean MAPKs are poorly understood.

In this study, I demonstrate the regulation mechanism of soybean MAPK under salt stress and pharmacological evidence of the GMK1 regulation mechanism and signaling pathway as well as translocation of GMK1 in soybean

2-1.4. Nuclear translocation of MAPK

Mitogen-activated protein kinase (MAPK) is activated various biotic abiotic stresses such as pathogen elicitor, virus, cold and osmotic stress. Salt stress activated MAPKs are also reported from many studies. AtMAPK4 and AtMPK6 are activated by salt in *Arabidopsis* (Ichimura et al., 2000), OsMPK4 in rice (Fu et al., 2002), SIMK in alfalfa (Munnik et al., 1999) and GMK1 in soybean (Fig. 2-1).

MAPK transfers signal to nucleus and regulates gene expression. In mammalian cells, most of transmission signal to nucleus is mediated by MAPK (Plotnikov et al., 2011), and the translocation is related with MAPK activity. This information is similar with plant MAPK. PcMPK6 and 3 are translocated to nucleus by ozone and the translocation is related by their activities because PcMPK4 is not translocated to nucleus, which is not activated by ozone (Lee et al., 2004).

2-2. Materials and methods

2-2.1. Plant material

Glycine max L. seeds were surface-sterilized with bleach solution (0.2% Chlorox) for 5 min, followed by five washes with sterilized distilled water. Seeds were placed on wet paper towels for germination in a growth chamber (25°C, 60% humidity) for 7 days under dark conditions. Before chemical treatments, all seedlings were stabilized in B&D solution (Broughton and Dilworth, 1971) for at least 4 h. After the treatment, seedling samples, excluding cotyledons and hypocotyls, were immediately frozen in liquid nitrogen and pulverized using a mortar and pestle. Ground samples were stored at -80°C until use.

2-2.2. Preparation of protein extracts and western blot analysis

Powdered tissue samples were added up to the 400 μl in a 1.5 ml tube containing 200μl of protein extraction buffer [50 mM Tris (pH 7.4), 1% NP-40, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM Na_3Vo_4, 1 mM NaF, and protease inhibitor cocktail]. The samples were incubated on ice for 5 min and homogenized. After centrifugation at 15,000 rpm for 15 min at 4°C, supernatants were transferred into new tubes. After two additional centrifugations, the concentrations of protein in the final supernatants were determined by the Bradford method. Thirty micrograms of total protein was used for SDS-PAGE and transferred onto a nitrocellulose membrane (PROTRAN, Germany). The membrane was blocked using blocking solution [5% non-fat dry milk in PBST (137 mM NaCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, 2.7 mM KCl, 0.05% Tween 20)] at room temperature and washed three times with PBST for 30 min. Primary antibody anti-GMK1 [epitope: FNPEYQQ] or GMK2 [LNPEYA] was added to the membrane and goat anti-rabbit IgG conjugated to horseradish peroxidase (Promega) was used as a secondary antibody, followed by chemiluminescence.

2-2.3. Immunoprecipitation (IP) and in-gel kinase assay

For the IP assay, total protein samples (400 μg) were incubated with anti-GMK1 antibody at 4°C for 2 h, and then precipitated with protein A-sepharose. After washing with a washing buffer [50 mM HEPES, pH 7,5, 5 mM EGTA, 2 mM EDTA, 2 mM DTT, 50 mM β-glycerophosphate, 10 mM NaF, and protease inhibitor cocktail], the beads were eluted with SDS sample buffer at 95°C for 3 min and subjected to an in-gel kinase assay. In-gel kinase assay was performed as described previously (Lee et al., 2008).

2-2.4. Total RNA isolation and northern blot analysis

Total RNA extraction and northern blot analysis were performed by following the methods described previously (Lee et al., 2008). Briefly, 20 μg of total RNA was separated on a 1.2% formaldehyde agarose gel and transferred to a nylon membrane. The membrane was hybridized with a gene-specific probe (GenBank accession number AF329506, nucleotide position 1004-1175) and washed with SSC buffer. After washing, the membrane was exposed to X-ray film.

2-2.5. Phospholipid isolation and thin-layer chromatography (TLC)

Phospholipid isolation was performed as described previously (Welti et al., 2002). Approximately 100 mg of pulverized samples were transferred to 0.01% butylated hydroxytoluene (BHT) containing 3 ml isopropanol at 75°C for 15 min. After the addition of 1.5 ml chloroform and 0.6 ml distilled water, the tubes were agitated for 60 min and the extracts were removed. Samples were re-extracted with 0.01% BHT containing chloroform: methanol mixture [2:1 (v/v)] after agitation for 30 min. This step was repeated 3 times. Remaining samples were heated until they were completely dried. The weights of the dried samples were used to establish the ratio of phospholipid/weight. The combined extracts were washed with 1 ml of 1 M KCl and once with 2 ml distilled water. The solvent was evaporated with nitrogen and the phospholipid extracts were dissolved in 1 ml chloroform. Isolated phospholipids were separated by TLC (Silica gel 60) with chloroform: methanol:

acetic acid: water mixture [85:15:12.5:3.5 (v/v)]. Phospholipid standards of PA, PC, and PE were purchased from Avanti Polar Lipids, Int. Separated phospholipids were made visible with iodine vapor and the intensities of the PA signals were measured using Multi gauge 4.0 (Fujifilm).

2-2.6. H_2O_2 and O_2^- measurement

Hydrogen peroxide content was measured using 5-(and-6)-chloromethyl-2,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA). Soybean seedlings were treated with 10 μM CM-H_2DCFDA (Molecular Probes^{TM}) for 60 min. After the seedlings had been treated with chemical inhibitors and/or NaCl, H_2O_2 signals were detected using the fluorescein isothiocyanate (FITC) channel of a fluorescence microscope (Carl Zeiss).

To detect O_2^-, 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT; Sigma, USA) was used. The chemical inhibitor or D.W.-treated soybean seedling roots were embedded in XTT and 300 mM NaCl solution, and then 200 μl of the solution was used for measuring XTT reduction using a spectrophotometer at an absorbance wavelength of 470 nm (A_{470}).

2-2.7. Protoplast isolation and polyethylene glycol transfection

The protoplast isolation method used in this study has been previously described (Yoo et al., 2007). Briefly, roots and hypocotyls of 7-day-old seedlings that were grown in dark conditions were cut to a size of 1 mm and transferred to an enzyme solution (1% w/v of cellulase RS (YAKULT, Japan) and macerozyme R-10 (MB cell, Korea), 0.4 M mannitol, 20 mM KCl and MES, 10 mM $CaCl_2$, and 0.1% BSA). After the solution was incubated at room temperature for 3 h, it was diluted with an equal volume of W5 solution (150 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, and MES; pH 5.7). The solution was then filtered through an 80 μm nylon mesh, centrifuged at 200 ×*g* for 3 min, and resuspended in W5 solution. W5 solution was removed and the cells were resuspended in MMG solution (0.4 M mannitol, 15 mM

$MgCl_2$, and 4 mM MES; pH 5.7). PEG transfection was performed as described previously (Yoo et al., 2007).

2-2.8. Immunolocalization assay

Paraffin-embedded roots were cut and fixed onto a slide glass, and the paraffin was removed by xylene. The samples were treated using ethanol for rehydration and washed with phosphate buffered saline Tween 20 (PBST; 137 mM NaCl, 1.5 mM KH_2PO_4, 2.7 mM KCl, 8 mM Na_2HPO_4, and 0.5 ml Tween 20). The slides were incubated in a blocking buffer [5% (w/v) non-fat milk powder in PBST] for 45 min and washed using PBST. After the slides were treated with the anti-GMK1 antibody and washed 3 times using PBST, an FITC-conjugated antibody was added. The slides were washed and the signal was measured using the z-stack method of a confocal microscope (LSM510, Carl Zeiss). 0.25 μg/ml of 4′,6-diamidino-2-phenylindole (DAPI) was used to detect nucleus.

2-2.9. Nuclei isolation and signal detection

Nuclei were isolated from ground soybean samples by PARTEC nuclei extraction buffer (Münster, Germany). Briefly, 400 μg of ground soybean root tissue was poured into 200 μl of the solution and incubated on ice for 20 min and filtered through 30 μm mesh. The filtrate was centrifuged at 10,000 x g for 15 min. The pellet was resuspended in 200 μl of PARTEC staining buffer. Anti-GMK1 antibody and FITC-conjugated antibody was added to the solution and washed with the staining buffer. GMK1 signal was detected with a confocal microscope (LSM510, Carl Zeiss) and the intensity was measured by Image J program and calculated total intensity/ detected nuclei number.

2-3. RESULTS

2-3.1. Salt stress activates GMK1 via post-translational regulation

To understand the role of salt stress-activated MAPKs in soybean, 7-day-

old dark-grown soybean seedlings were treated with various concentrations of NaCl for 5 min. In-gel kinase assays showed that a 47-kD MAPK activity was induced upon treatment with increasing concentrations of NaCl, up to 300 mM (Fig. 2-1A).

In our laboratory, we have cloned six soybean MAPKs from the public soybean EST database. Among these, GMK1 [*Gm*MPK6] was thought to be as a putative candidate for a salt stress-activated MAPK, because this MAPK is orthologous to SIMK and AtMPK6 (Daxberger et al., 2007), which are activated by salt (Munnik et al., 1999; Ichimura et al., 2000). To confirm that the 47-kD MAPK corresponds to GMK1, IP and in-gel kinase assay were performed with a GMK1-specific antibody (Fig. 2-1B). The specificity of the anti-GMK1 antibody was tested with recombinant GMK1 protein expressed in *Escherichia coli* (data not shown). Kinase activity was detected only in the immunoprecipitates derived from seedlings that had been treated with 300 mM NaCl, indicating that the salt stress-activated 47-kD MAPK was indeed GMK1. This result was also confirmed by immunodepletion assay (Fig. 2-1D).

To understand the mechanism of GMK1 regulation under salt stress, expression of GMK1 RNA and protein levels were measured after NaCl treatment by northern and western analyses. RNA and protein levels of GMK1 were not changed in any of the NaCl treatment conditions (Fig. 1-1C).

For the time course analysis of GMK1 activity, soybean seedlings were treated with 300 mM NaCl for up to 120 min. GMK1 activity was induced very rapidly, as early as 5 min, and consistently increased until 10 min. The activity was sustained up to 30 min of the treatment, and then declined (Fig. 2-2A). However, gene and protein levels were not changed under these conditions (Fig. 2-2B and C). These results suggest that the activity of GMK1 is regulated by a post-translational mechanism.

Many salt stresses signaling pathways are corresponded with osmotic stress. Therefore, GMK1 activation was examined in mannitol and ABA treatment. 300 mM NaCl, 300 mM mannitol and 50 μM ABA were treated to soybean

seedlings for 5 min, respectively and GMK1 activities were measured from the seedlings. In mannitol treatment, GMK1 activity was weaker than NaCl treatment and more reduced in ABA treatment (Fig. 2-3). This result suggested that GMK1 is activated by osmotic stress, but its regulation mechanism is different with that of salt stress.

2-3.2. GMK1 activity is regulated by PA via heteromeric G-protein, PLD, and PLC under salt stress conditions

There are several reports on G-protein-mediated plant MAPK activation. In *Arabidopsis*, AtMPK6 is activated by mastoparan, a heterotrimeric G-protein activator (Miles et al., 2004). In rice, OsMAPK6 activity is diminished in the null mutant for the gene encoding the α subunit of the heterotrimeric G-protein (Lieberherr et al., 2005). These two MAPKs are probably orthologus of soybean GMK1. To determine whether mastoparan has the ability to activate GMK1, 7-day-old soybean seedlings were treated with 5 μM mastoparan for 30 min, and then carried out an in-gel kinase assay. As shown in Fig. 2-4 A, the 47-kD MAPK was activated by mastoparan, and IP data further revealed that the activated MAPK is indeed GMK1 (Fig. 2-4B).

Downstream effectors of heterotrimeric G-protein are diverse and include PLC and PLD (Ullah et al., 2003). Accordingly, It could be hypothesized that soybean G-protein could activate GMK1 via a signaling pathway involving PLD and PLC. In order to reveal the relationship between GMK1 and G-protein downstream effectors, the soybean seedlings were treated with inhibitors of PLD or PLC, 0.1% *n*-butanol or 15 μM neomycin, respectively, for 60 min, followed by treatment with 5 μM mastoparan. As shown in Fig. 2-4 C, the in-gel kinase assay revealed that the mastoparan-inducible GMK1 activity was decreased by *n*-butanol or neomycin treatment. These results suggest that GMK1 was activated by mastoparan via the PLD and PLC pathways.

In order to determine whether heteromeric G-protein and its effectors also

participate in the salt stress signaling pathway, soybean seedlings were treated with 10 μM suramin for 60 min and transferred them to 300 mM NaCl for 5 min. Suramin is a known heterotrimeric G-protein inhibitor in mammalian cells (Olivier et al., 1990) and an inhibitor of MAPK activity induced by oxidative stress in tobacco suspension-cultured cells (Miles et al., 2002). As shown in Fig. 2-4 D, GMK1 activation, induced by 300 mM NaCl, was decreased by suramin. Similar experiments with *n*-butanol and neomycin also showed diminished GMK1 activity after 5 min of NaCl treatment (Fig. 2-4E). These results suggest that components and effectors of the salt stress signaling pathway are shared with mastoparan signaling.

Since PA is a product of PLD and PLC activity, I isolated phospholipids from 300 mM NaCl-treated soybean seedlings and separated them by TLC to measure PA generation under salt stress (Fig. 2-5). Endogenous PA level was increased after 5 min of treatment with 300 mM NaCl, but decreased after 10 min (Fig. 2-6A).

To confirm the relationship between PA and GMK1 activation, soybean seedlings were treated with 50 μM of exogenous PA for 5 min and subjected to an in-gel kinase assay. As shown in Fig. 2-6 B, the 47-kD MAPK was activated by PA, which was identified as GMK1 by IP (Fig. 2-6C). Taken together, our data suggest that GMK1 activity is regulated by PA via heteromeric G-protein, PLD and PLC in the early stages of the response to salt stress.

2-3.3. Reactive oxygen species were generated in salt-treated soybean roots

Aforementioned data suggested that the PA signaling controls GMK1 activity up to 5 min of NaCl treatment. However, salt-inducible GMK1 activity was persistent up to 30 min. Therefore, it is reasonable to speculate that another modulator has a role in regulation at the latter stage of salt stress.

Reactive oxygen species (ROS) is a well-characterized MAPK modulator. Salt stress has known to induce oxidative bursts (Katsuhara et al., 2005). To

examine ROS generation in soybean root by NaCl treatment, XTT and CM-H_2DCFDA were used to detect superoxide (O_2^-) and H_2O_2, respectively. As shown in Fig. 2-7 A, O_2^- increased continuously with 300 mM NaCl treatment. Hydrogen peroxide also increased, beginning at the secondary root tip by 3 min, spreading throughout to peripheral region of the tip by 5 min, and then to other regions of the secondary roots by 30 min. Finally, H_2O_2 was generated in all regions of the secondary root by 60 min after the treatment (Fig. 2-7B). This phenomenon was also detected in whole seedling root (data not shown).

To examine whether NADPH-oxidase is the source of ROS production, the production was tested with/without an NADPH-oxidase inhibitor, diphenyleneiodonium (DPI). H_2O_2 production was significantly reduced compared to the control (Fig 2-8). These results suggest that ROS generation by high salts in soybean roots is produced by NADPH-oxidase.

2-3.4. Hydrogen peroxide activates GMK1 independently of PA

To examine whether ROS activates GMK1 or not, soybean seedlings were treated with in 1 mM H_2O_2 for 60 min and activity was measured using myelin basic protein (MBP) as a MAPK substrate. H_2O_2 strongly activated 47-kD MAPK as early as 3 min after treatment, and its activity was maintained for up to 60 min (Fig. 2-9A). In soybean, GMK1 and GMK2 (GmMPK3) are orthologus of AtMPK6 and AtMPK3, respectively. The two MAPKs are activated by H_2O_2 in *Arabidopsis*. To further reveal the identity in soybean, immunoprecipitation and an in-gel kinase assay were performed with these two kinds of MAPK antibodies, respectively. The anti-GMK1 antibody-precipitated MAPK was activated by H_2O_2 treatment (Fig. 2-9B), suggesting that GMK1 is activated by H_2O_2.

GMK1 was activated by PA (Fig. 2-6). Therefore, it is reasonable to speculate that GMK1 activation is regulated by both PA and H_2O_2. To examine whether GMK1 activation by H_2O_2 is mediated by PA, soybean seedlings were treated with *n*-butanol or neomycin for 60 min and then treated with 1 mM H_2O_2 for

5 min. Both chemicals did not affect GMK1 activity induced by H_2O_2 (Fig. 2-9C), suggesting that H_2O_2 activates GMK1 independently of PA though PLD and PLC.

2-3.5. Activity of GMK1 is regulated by PA and hydrogen peroxide at different time points in salt stress

Previous studies indicate that both PA and ROS activate GMK1. In salt stress, PA reached its maximum level at 5 min (Fig. 2-6) but H_2O_2 was significantly increased even after 30 min of NaCl treatment (Fig. 2-7B). Therefore, it could be speculated that PA and H_2O_2 activate GMK1 with different time points in salt stress. To investigate the effect of endogenous ROS on GMK1 activity, soybean seedlings were treated with DPI for 60 min and then treated with 300 mM NaCl for up to 240 min. DPI treatment decreased GMK1 activity from 10 min after treatment (Fig. 2-10A). This result suggested that NADPH-oxidase dependant ROS was likely to be involved in maintaining GMK1 activity from 10 min of the NaCl treatment.

For a detailed examination of GMK1 activity regulated by PA under salt stress, PLD and PLC dependant GMK1 activity was monitored at early time points. Activity of GMK1 induced within 1 min was gradually increased till 5 min of 300 mM NaCl treatment (Fig. 2-10B). The *n*-butanol pre-treatment, however, reduced GMK1 activity from 5 after the treatment. Especially, GMK1 activity at 5 min was strongly affected by *n*-butanol (Fig. 2-10C). Neomycin treatment also reduced the activity in a time-dependant manner, with a strongest reduction at 3 min after NaCl treatment (Fig. 2-10D). These results suggested that GMK1 activity in the initial 5 min of NaCl treatment was regulated by the PA.

Based on these results, I propose that GMK1 activity may be induced by PA though PLD and PLC during early stages of salt stress but maintained by H_2O_2 at later stages.

2-3.6. Reactive oxygen species generation is indirectly regulated by GMK1

Previous reports suggest the activation of MAPK by ROS, but ROS

generation is also regulated by MAPK (Pitzschke and Hirt, 2009). To elucidate the relationship between GMK1 activation and ROS generation under salt stress, I used SB202190, a specific inhibitor of p38 MAPK, to inhibit GMK1 activity and measured ROS generation. GMK1 activity was decreased by the SB202190 application in NaCl treatment (Fig. 2-11A). Super oxide generation detected by XTT in NaCl-treated seedlings increased slightly until 120 min after the treatment compared to control seedlings, and then reduced (Fig. 2-11B), suggesting indirect effect of GMK1 on ROS generation. To further examine this relationship, GMKK1 (GenBank accession No.: BF598140) was expressed to protoplast generated from soybean root as a constitutively activated form. GMKK1 activated GMK1 *in vitro* (data not shown). Constitutively activated GMKK1 was manufactured by substituting two amino acids (T_{211}→E and S_{217}→D; hereafter, TESD-GMKK1). TESD-GMKK1 was transfected into protoplasts generated from soybean roots and hypocotyls using PEG transfection method. After incubation for 10 h, protoplasts were treated with XTT and incubated for another 2 h. TESD-GMKK1-transfected protoplasts generated more O_2^- than vector-transfected protoplasts (Fig. 2-11C). However, the XTT-formazan signal was very weak. After additional 8 h of incubation, TESD-GMKK1–transfected protoplasts showed 3 times higher generation of O_2^- than the vector control (Fig. 2-11D). These data also suggest that ROS generation is indirectly regulated by GMK1 under salt stress.

2-3.7. Total SOD activity in 300 mM NaCl treatment

NADPH-oxidase localizes in plasma membrane and generates superoxide to outside of plasma membrane. Superoxide directly changed to hydrogen peroxide by superoxide dismutase (SOD). Therefore, SOD activity is very closely related with H_2O_2 generation ratio. To identify SOD activity in NaCl treatment, I treated 300 mM NaCl to soybean seedlings for 30 min and 60 min, respectively. SOD activities were measured in non-denaturated acrylamide gel. As shown in Fig. 2-12, total SOD activity was not changed in 300 mM NaCl treatment until 60 min. This

result suggested that hydrogen peroxide generation ratio is not affect SOD activity in 300 mM NaCl treatment.

2-3.8. Morphological analysis of soybean in salt stress with GMK1 signaling pathway inhibitors

Salt stress affects physiology and metabolism of plant. 300 mM NaCl is too high to measure the effects of chemical in salt stress, because soybean shoot and root hardly grown in the concentration (Umezawa et al., 2000; Munns and Tester, 2008). Therefore, effects of GMK1 signal inhibitors on soybean seedlings were measured in 150 mM NaCl. To identify the physiological effect of GMK1 signaling pathway related inhibitors in salt stress, 10-days old soybeans were treated with 150 mM NaCl, as well as co-treated with 150 mM NaCl and 1% *n*-butanol, 150 mM NaCl and 50 μM DPI, and 150 mM NaCl and 30 μM SB202190, respectively. After 5-days of the treatment, I observed the soybean's shapes. As shown in Fig. 2-14, 150 mM NaCl treated soybean was less grown than control soybean grown in B&D solution. 150 mM NaCl treated soybean leaves were etiolated and rimpled. Moreover, the shape of 150 mM and inhibitor treated soybean was also similar to that of 150 mM NaCl treated soybean.

To analyze the effect of inhibitors on 150 mM NaCl treatment, I measured root and shoot length of the soybean and checked leaves and stem color. The root length of 150 mM NaCl treated soybean was shorter than normal soybean. However, 150 mM NaCl + *n*-butanol, 150 mM NaCl + SB202190 and 150 mM NaCl + DPI treated soybean roots were longer than only 150 mM NaCl treated soybean root, respectively (Fig. 2-15A).

This morphology is not correlated with shoot length. 150 mM NaCl treated soybean shoot was shorter than that of control soybean. 150 mM NaCl + *n*-butanol, 150 mM NaCl + SB202190 and 150 mM NaCl + DPI treated soybeans were shorter than 150 mM NaCl treated soybean, respectively (Fig. 2-15B). These results indicated that GMK1 signaling related inhibitors exerted more influence on shoot

length than root length.

In 150 mM NaCl condition, soybean leaf and stem color was etiolated (Fig. 2-16). 150 mM NaCl + *n*-butanol and 150 mM NaCl + SB202190 treated soybean leaves colors were similar with only 150 mM NaCl treated soybean leaf, however DPI treated soybean leaves were more seriously etiolated than other treatment (Fig. 2-16A). Stem color also changed. B&D solution grown soybean stem appear as green. However, 150 mM NaCl treated soybean stem colors violet, indicating that salt stress likely cause anthocyanin accumulation. 150 mM NaCl and other inhibitor treated soybean stem also same color with 150 mM NaCl treated soybean stem, except *n*-butanol treated soybean (Fig. 2-16B). 150 mM + *n*-butanol treated soybean stem was completely etiolated. These results suggested that DPI and *n*-butanol differently regulated leaf and stem color in salt stress.

Salt stress induces various genes or reduces genes expression. To identify gene expression regulated by GMK1 in salt stress, soybean seedlings were treated with 30 μM SB202190 for 1 h and then treated with 300 mM NaCl for 3 h. As shown Fig. 2-17 B, gene expression of *GmNADPH-oxidase* was slightly increased in 300 mM NaCl, but decreased by SB202190 treatment. Expression of *GmRD19* and *GmRD21* was decreased by NaCl treatment, but slightly increased from the reduction by SB202190 treatment. The GmRD19 and GmRD21 are homologus of *RD19* and *RD21* in *Arabidopsis*, respectively, and the expression of *GmNADPH-oxidase* is already reported in salt stress. This result suggested that activity of GMK1 affects to various salt-related genes expression in salt stress.

2-3.9. GMK1 is translocated to nucleus by NaCl treatment.

Many mammalian MAPKs are translocated to nucleus and transfer the signal as well as regulate gene expression (Plotnikov et al., 2011). Moreover, part 1 data suggest that GMK1 indirectly regulates ROS generation (Fig. 2-11). In this context, it is possible that GMK1 transfers to nucleus and regulates expression of ROS generation-related genes in salt stress. Therefore, I investigated translocation

of salt stress-activated GMK1 with immunodetection assay. Under normal condition, GMK1 was localized in cytosol or near the plasma membrane (Fig. 2-18A) and the localization was not changed in 10 min and 30 min of 300 mM NaCl treatment (data not shown). However, its signal was detected in nucleus after 60 min of the treatment (Fig. 2-18B). To elucidate this phenomenon more, I isolated nuclei and investigated GMK1 localization in nucleus with anti-GMK1 antibody. GMK1 signals were not detected in normal seedling nuclei, but detected in NaCl treated seedlings nuclei (Fig. 2-18C and D). This result suggests that GMK1 is translocated to nucleus by NaCl treatment.

2-3.10. Nuclear translocation of GMK1 is reduced by MAPK inhibitor.

Nuclear translocation of MAPKs is occurred after phosphorylation of their active site. This means that activity of MAPK is very important for the translocation. To determine whether nuclear translocation of GMK1 is affected by its activity or not, soybean seedlings were treated with 30 μM of SB202190, a MAPK inhibitor prior to the treatment with 300 mM NaCl for 60 min. SB202190 reduced GMK1 activity under salt stress (Fig. 2-11A). The seedlings were longitudinally cut and treated with anti-GMK1 antibody and FITC-conjugated antibody. The samples were compared with samples only treated with 300 mM NaCl as a control. Most of GMK1 signals were matched with DAPI signal in 300 mM NaCl treatment (Fig. 2-19A). However, signals of GMK1 by NaCl treatment were not corresponded to the nucleus signal by SB202190 treatment (Fig. 2-19B).

GMK1 signals were also reduced by SB202190 treatment in nuclei of salt-treated seedlings. Nuclei were isolated from control, seedlings treated with 300 mM NaCl and seedlings treated with SB202190 after 300 mM NaCl treatment, respectively. GMK1 signals were detected with a confocal microscopy and measured the signal intensity. In 300 mM NaCl treated seedlings, GMK1 signals in nucleus were increased compared to control seedling, but the signals were decreased by SB202190 treatment (Fig. 2-19C). These results suggest that GMK1

translocation is affected by its reduced activity by a MAPK inhibitor.

2-3.11. Nuclear translocation of GMK1 is induced by hydrogen peroxide.

In aforementioned data, GMK1 is dually activated by PA and H_2O_2 and translocated to nucleus under salt stress. Moreover, PA also constantly activated GMK1 as H_2O_2 constantly did (Fig. 2-9). This data suggest that PA and H_2O_2 could be plausible candidates to regulate nuclear translocation of GMK1 under salt stress. To investigate whether GMK1 is translocated to nucleus by these two molecules or not, soybean seedlings were treated with PA and H_2O_2 for 60 min, respectively. GMK1 signals were barely matched to nucleus signals from PA treatment (Fig. 2-20A). However, GMK1 signals were strongly concentrated and corresponded to DAPI signals in H_2O_2 treatment (Fig. 2-20B). To further reveal nuclear translocation of GMK1, we isolated nuclei from the samples and detected GMK1 signals in the nuclei. GMK1 signals were detected in the nuclei from seedlings treated with H_2O_2, but not in the nuclei from seedlings treated with PA (Fig. 2-20C and D). This result strongly suggests that nuclear translocation of GMK1 is regulated only by H_2O_2.

2-3.12. Nuclear translocation of GMK1 is mediated by endogenous hydrogen peroxide in salt stress.

Although GMK1 was translocated to nucleus by exogenous H_2O_2, it could not be direct evidence of the function of endogenous H_2O_2 on nuclear translocation of GMK1 under salt stress. Moreover, function of endogenous PA on nuclear translocation of GMK1 also has not been studied yet. Therefore, nuclear translocation of GMK1 by 300 mM NaCl treatment by blocking PA generation under salt stress was investigated.

To reduced PA generation under salt stress, soybean seedlings were treated with *n*-butanol for 60 min and then treated with 300 mM NaCl for 60 min. In the samples, GMK1 signals were not changed by *n*-butanol (Fig. 2-21A), and also strongly detected in isolated nuclei from the seedlings (Fig. 2-21C). Therefore, *n*-

butanol could not affect nuclear translocation of GMK1 under NaCl treatment.

To investigate function of endogenous H_2O_2 on GMK1 translocation under salt stress, soybean seedlings were treated with 50 μM DPI for 60 min prior to the treatment with 300 mM NaCl for 60 min. Even though plasmolysis symptom was also observed in the seedlings, GMK1 signals were not corresponded to the nucleus. Its signal covered all of the plasmolysed protoplasm (Fig. 2-21B). This data is more clearly shown in supplementary Figure S2. This result is similar to the data obtained from the isolated nuclei. GMK1 signals in the nuclei were not only diminished by DPI treatment but also detected only on the surface of nuclei (Fig. 2-21D). These results strongly suggest that translocation of GMK1 to nucleus is regulated by endogenous H_2O_2 under salt stress.

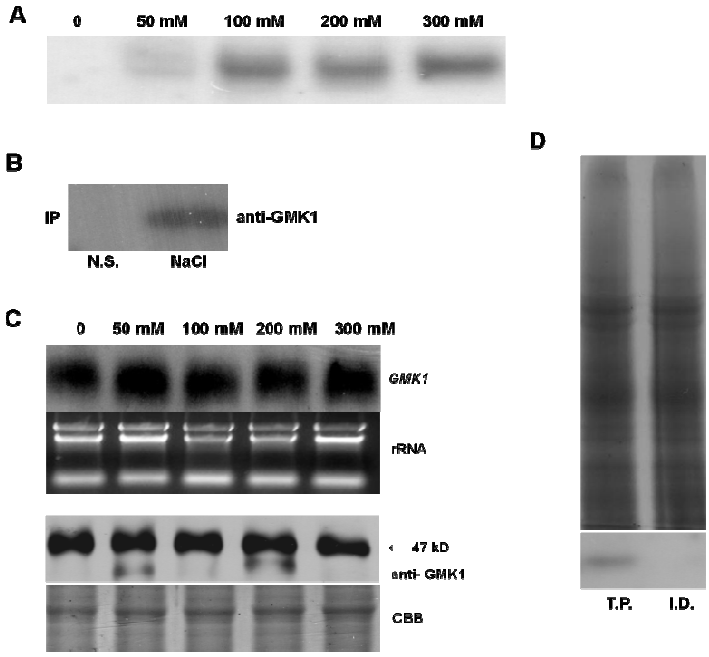


Figure 2-1. Activity of GMK1 was increased by salt concentration, but mRNA and protein levels are not changed. A. Total protein extracted from 7-day-old soybean seedlings that had been treated with various concentrations of NaCl (0–300 mM) for 5 min and subjected to an in-gel kinase assay. B. Immunoprecipitation (IP) with anti-GMK1 antibody (anti-GMK1) of total protein from soybean seedlings, either non-salt treated (N.S.) or treated with 300 mM NaCl for 5 min. C. Total RNA and protein were extracted from the same seedlings as in A and subjected to northern and western blotting with a *GMK1*-specific probe and anti-GMK1 antibody, respectively. MBP (myelin basic protein) was used as an artificial MAPK substrate. D. Total protein was isolated from 300 mM NaCl treated seedlings and subjected to immunodepletion and in-gel kinase assay. T.P. and I.D. stand for total protein and GMK1-immunodepleted total protein, respectively.

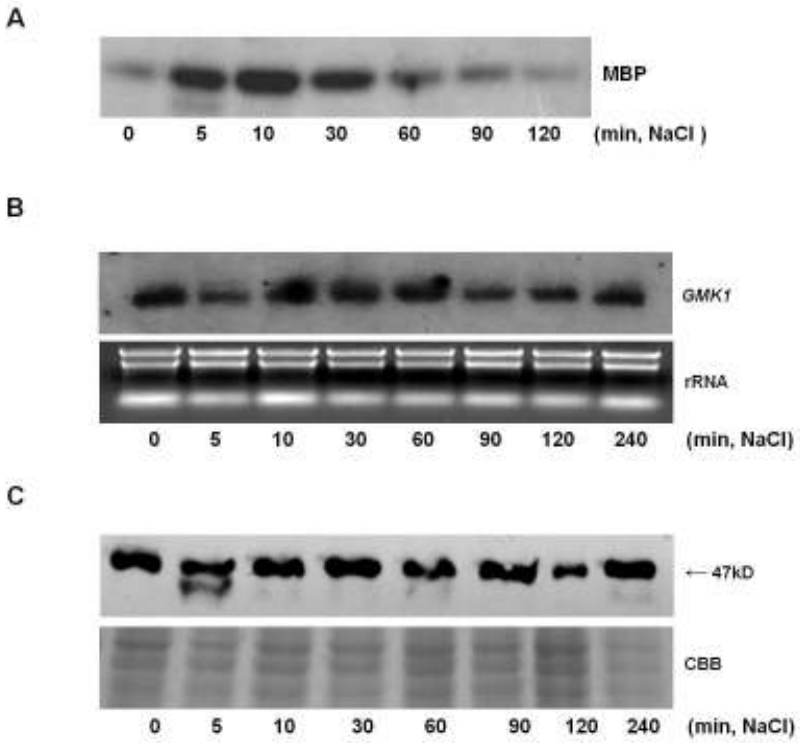


Figure 2-2. Time course analysis of GMK1 in 300 mM NaCl-treated soybean seedlings. A. Soybean seedlings were treated with 300 mM NaCl for 120 min. Total protein was extracted from the seedlings and subjected to an in-gel kinase assay. B. Soybean seedlings were treated with 300 mM NaCl for up to 240 min and total RNA was extracted. Northern blot analysis was performed with a *GMK1*-specific probe. C. Total protein was extracted from the same seedlings as in B, and western blot was performed with an anti-GMK1 antibody.

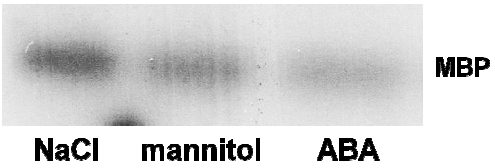


Figure 2-3. 47 kD MAPK was activated by salt and osmotic stresses. Soybean seedlings were treated with 300 mM NaCl, 300 mM mannitol and 50 μM ABA for 5 min, respectively. Total proteins were isolated from the seedlings and subjected to in-gel kinase assay.

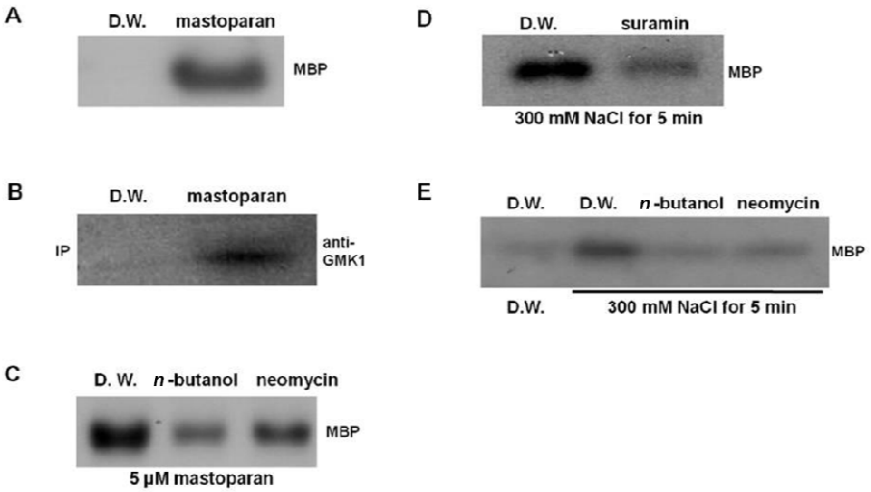
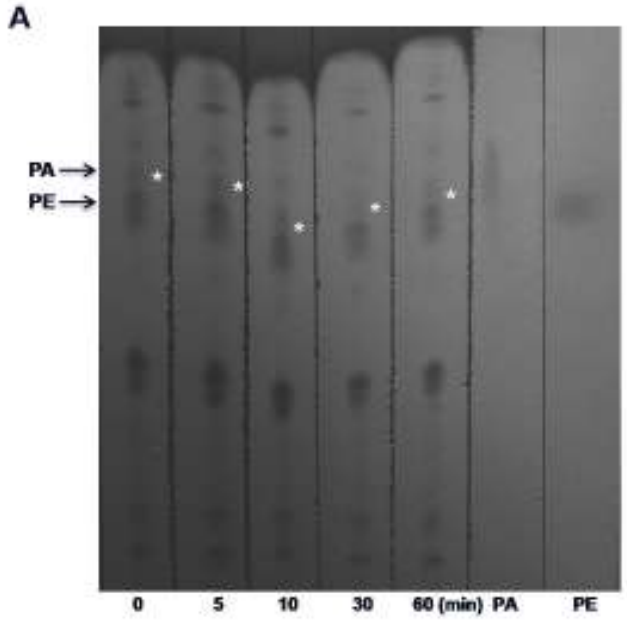


Figure 2-4. GMK1 was regulated by heteromeric G-protein and two phospholipases. A. Soybean seedlings were treated with 5 μM mastoparan for 30 min and were subjected to an in-gel kinase assay. B. Total protein extracted from mastoparan-treated samples was subjected to IP with an anti-GMK1 antibody. C. The seedlings were treated with *n*-butanol or neomycin for 60 min before mastoparan treatment, and then subjected to an in-gel kinase assay. D. Soybean seedlings were treated with suramin for 60 min, and then with 300 mM NaCl for 5 min. Total protein from these seedlings was subjected to an in-gel kinase assay. E. The seedlings were treated with *n*-butanol or neomycin for 60 min, then with 300 mM NaCl for 5 min, and then subjected to an in-gel kinase assay.



	R_f of PA	R_f of PE
0 min	0.77	0.71
5 min	0.77	0.71
10 min	0.75	0.70
30 min	0.75	0.70
60 min	0.76	0.71

Figure 2-5. Phospholipids were separated by TLC. A. Soybean seedlings were treated with 300 mM NaCl for various time points up to 1 h and phospholipids were extracted from these seedlings. Total phospholipid was separated by TLC and stained with iodine vapor. Shown is a representative image of three biological repeats. B. R_f of PA and PE. PA, phosphatidic acid; PE, phosphatidyl ethanolamine. B. R_f of PA and PE are shown in indicated lane.

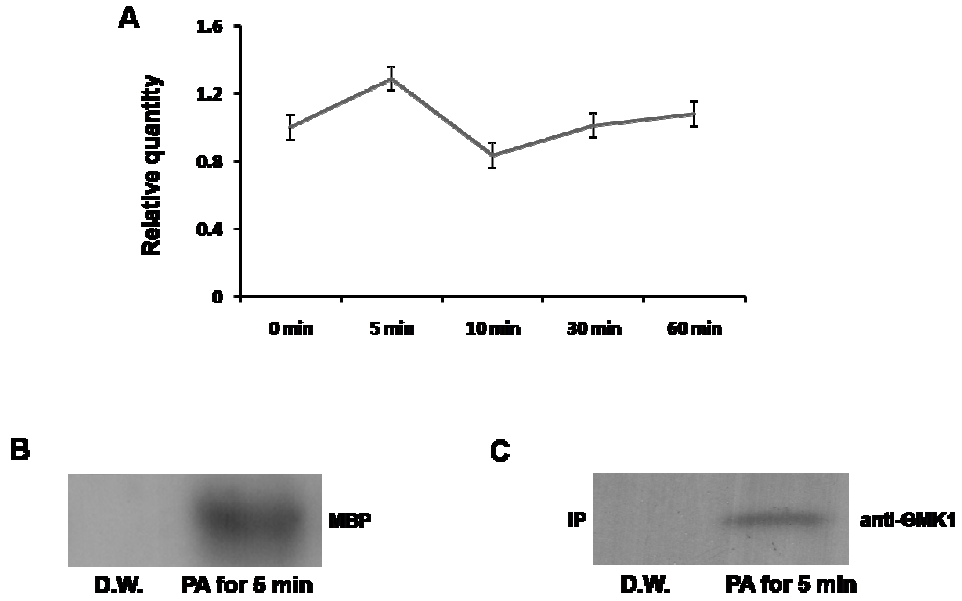


Figure 2-6. Endogenous PA is increased by 300 mM NaCl treatment at 5 min and exogenous PA activated GMK1 in soybean. A. Phospholipids isolated from soybean seedlings treated with 300 mM NaCl for the indicated time periods were separated by TLC and stained with iodine. The amount of PA was measured by Multi gauge 4.0 (Fujifilm). Mean ± SD of 3 samples. B. Soybean seedlings were treated with 50 μM PA for 5 min and then subjected to an in-gel kinase assay. C. Total protein extracted from PA-treated samples was subjected to IP and in-gel kinase assay with anti-GMK1 antibody.

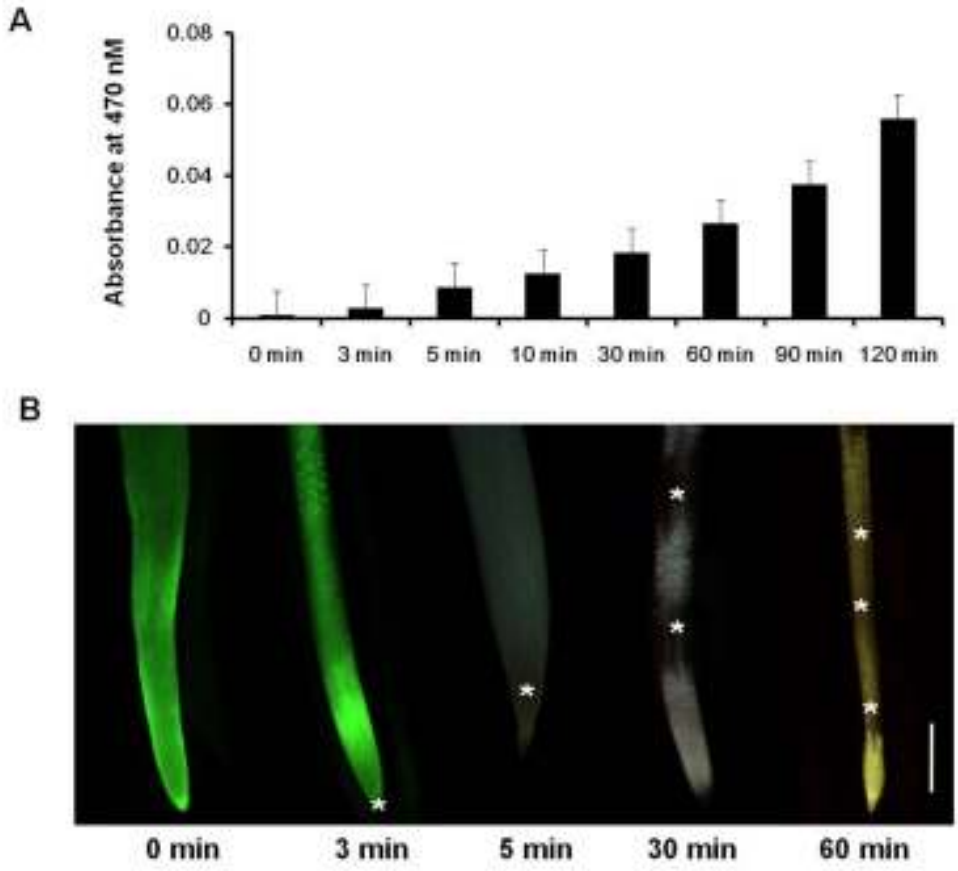


Figure 2-7. ROS generation in soybean root during NaCl treatment. A, Soybean seedlings were treated with 300 mM NaCl for the indicated times, and then O_2^- generation was measured using XTT-formazan with spectrophotometer at 470 nM. Mean ± SD of 2 samples. B, Soybean seedlings were treated with 10 μM CM-H_2DCFDA for 60 min, followed by 300 mM NaCl treatment for the indicated times. H_2O_2 generation signals in secondary roots were detected using the FITC channel of a fluorescence microscope. (*) indicates H_2O_2 signal. Scale bar = 1 mm.

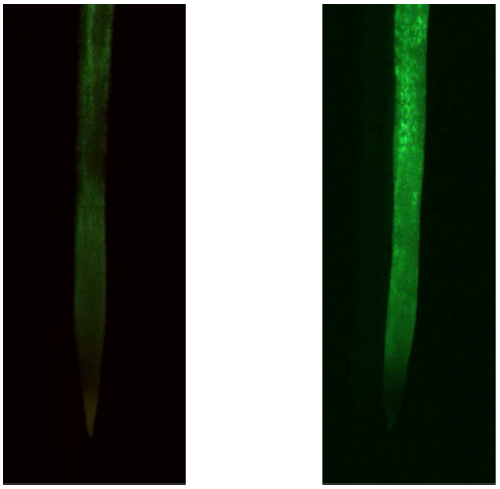


Figure 2-8. The NADPH oxidase inhibitor diminished NaCl-activated H_2O_2 generation. Soybean seedlings were treated with 50 uM DPI for 1 h before treatment with 300 mM NaCl for 30 min. H_2O_2 generation was detected with CM-H_2DCFDA. Control seedlings were treated with D.W. instead of DPI.

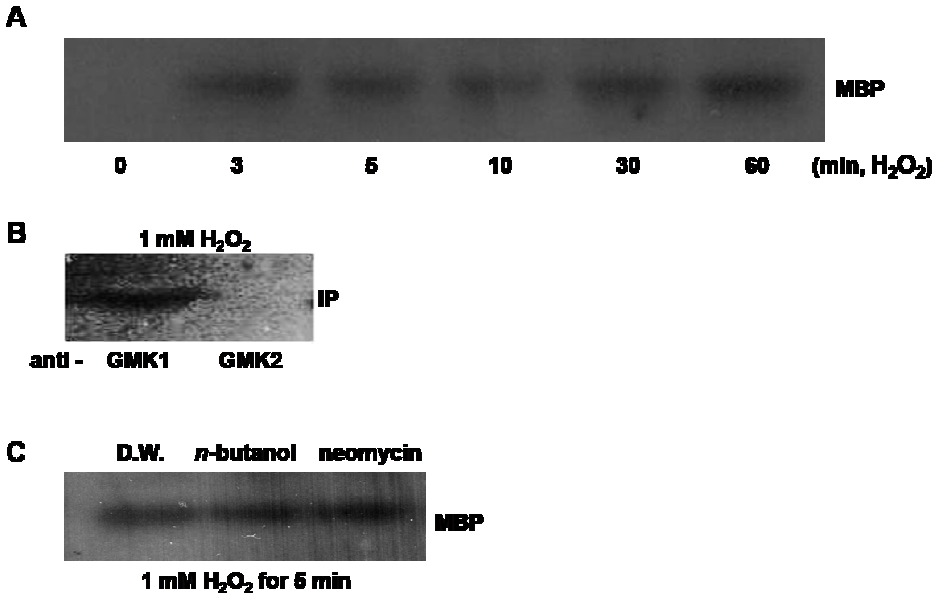


Figure 2-9. **H_2O_2 activated GMK1 independently PLD and PLC.** A, Soybean seedlings were treated with 1 mM H_2O_2 for indicated time points and subjected to an in-gel kinase assay. B, Total protein was extracted from 5 min sample of A and subjected to immunoprecipitation and an in-gel kinase assay using anti-GMK1 and anti-GMK2 antibodies. C, Seedlings were treated with 1% *n*-butanol or 15 μM neomycin for 60 min before treatment with 1 mM H_2O_2 for 5 min. Total protein extracted from these seedlings was subjected to an in-gel kinase assay. Myelin basic protein (MBP) was used as a MAPK substrate.

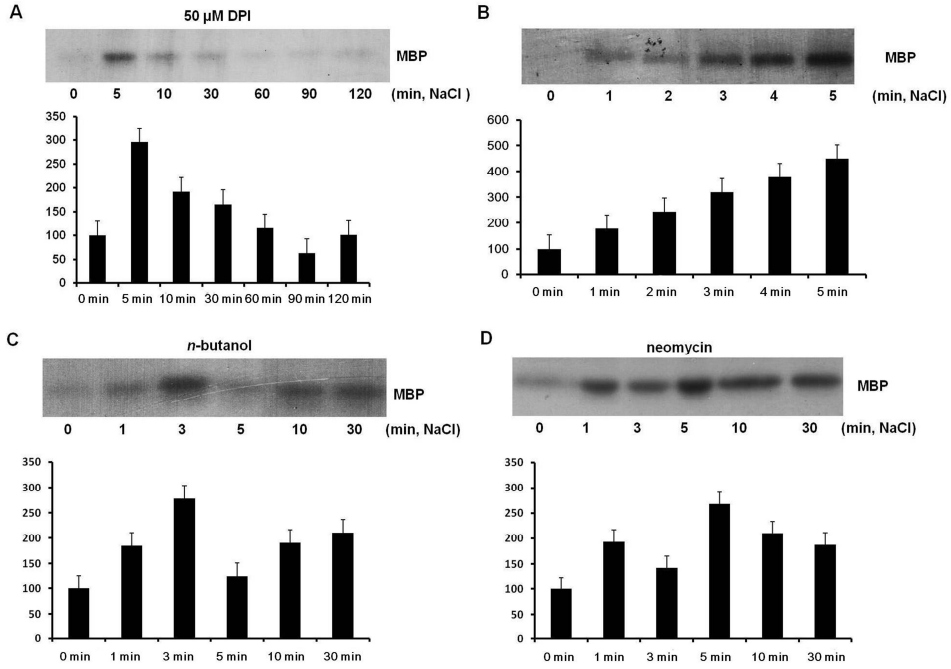


Figure 2-10. DPI and PA generation inhibitors reduced GMK1 activity at different time points during NaCl treatment. A, After soybean seedlings were treated with 50 μM DPI for 60 min, 300 mM NaCl was introduced to the seedlings at the indicated times, followed by an in-gel kinase assay. B, Soybean seedlings pre-treated with 300 mM NaCl at the indicated times; total protein from the seedlings was subjected to an in-gel kinase assay. C, Soybean seedlings were treated with 1% *n*-butanol for 30 min, then treated with 300 mM NaCl for the indicated times and subjected to an in-gel kinase assay. D, Treatment is the same as for C except 15 μM neomycin replaced *n*-butanol. All of the MAPK activities were measured by relative intensity of the band compared to control. The intensity was measured by LAS-3000 and Multi gauge 4.0 (Fujifilm, Japan). Control band intensity was set as a 100. Mean ± SD of 2 samples.

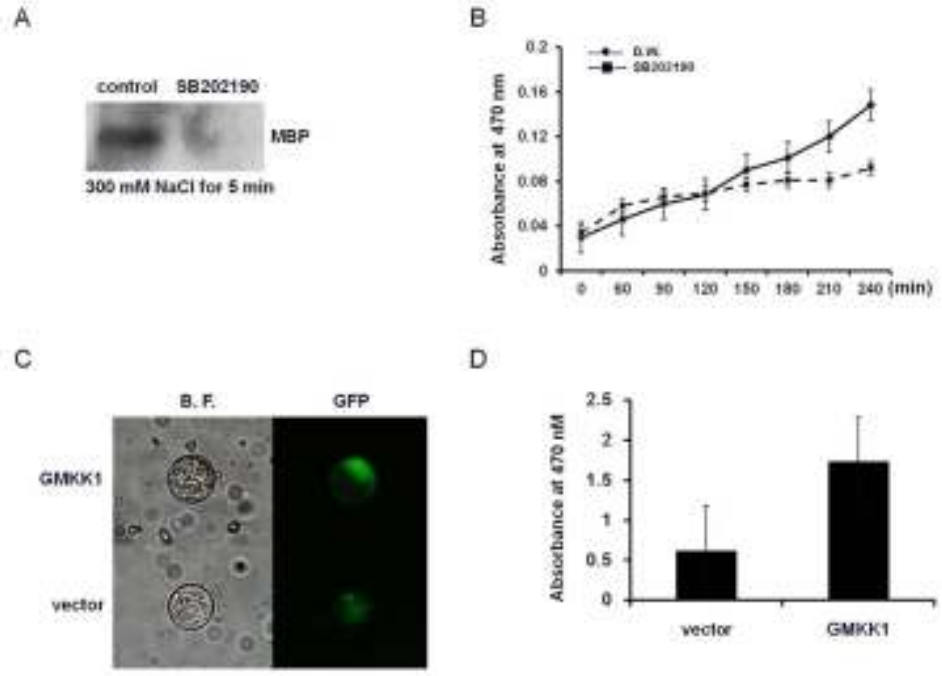


Figure 2-11. The effects of SB202190 and constitutively activated GMKK1 on ROS generation during salt stress. A, After soybean seedlings were treated with 30 μM SB202190 for 60 min, they were treated with 300 mM NaCl for 5 min. Total protein extracted from these seedlings was subjected to an in-gel kinase assay. B, SB202190-treated soybean seedlings were submerged in solution containing 300 mM NaCl and XTT for the indicated times. Absorbance of the solutions was measured at the time points. Mean ± SD of 4 samples. C, Constitutively activated soybean MAPKK, GMKK1, was cloned into a JJ2053 vector and transfected into the soybean protoplast using PEG transfection. After protoplasts were incubated for 10 h for gene expression, 0.5 mM XTT was for 2 h and XTT-formazan was detected using microscopy. D, XTT-formazan was also measured at A_{470} after 10 h incubation. B.F. indicates bright field. Mean ± SD of 2 samples.

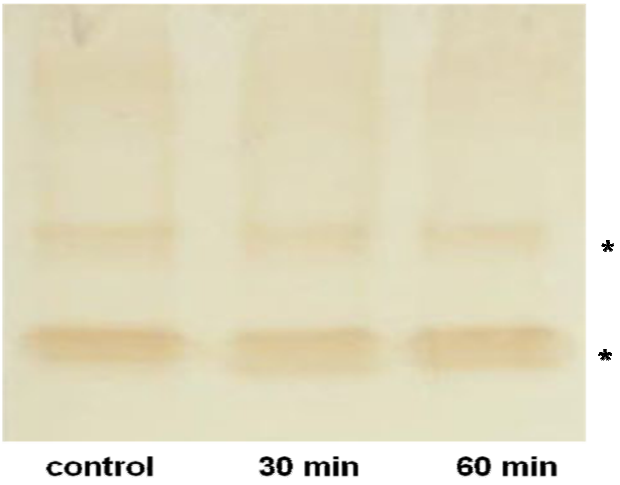


Figure 2-12. SOD activity in soybean seedlings treated with 300 mM NaCl. Total protein extracted from the seedlings was separated using a nondenaturing gel, and SOD activity was measured by previously described method (Misra and Fridovich, 1977). Control seedlings were treated with distilled water. (*) indicates SOD activity.

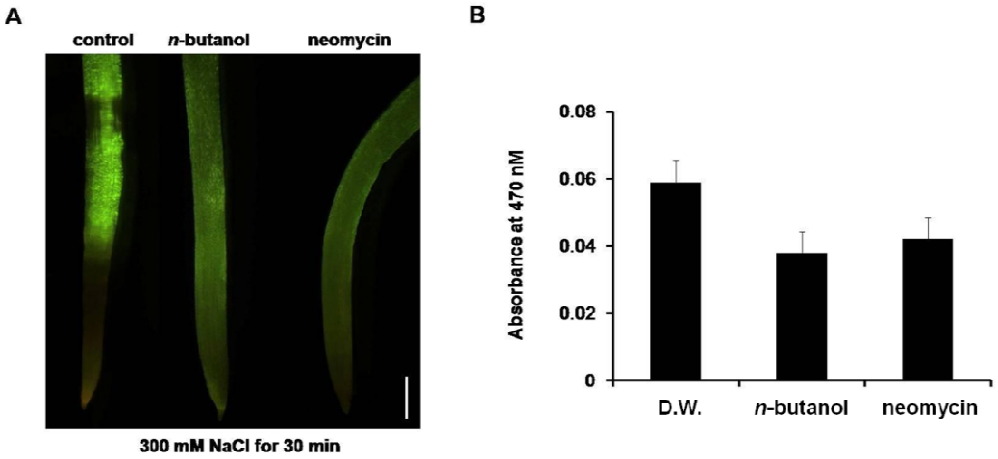


Figure 2-13. Reduction of ROS generation by PA production inhibitors in salt stress. A, Soybean seedlings were treated with CM-H_2DCFDA for 1 h before treatment with *n*-butanol and neomycin for 1 h. After these seedlings were imbedded in 300 mM NaCl for 30 min, H_2O_2 generation was detected with fluorescence microscope. B, Soybean seedlings were treated with n-butanol and neomycin for 1 h before co-treatment with 300 mM NaCl and 0.5 mM XTT. After 90 min treatment, XTT formazans were detected with spectrophotometer at A_{470}. D.W. treatment was used as a control of inhibitors. Mean ± SD of 3 samples

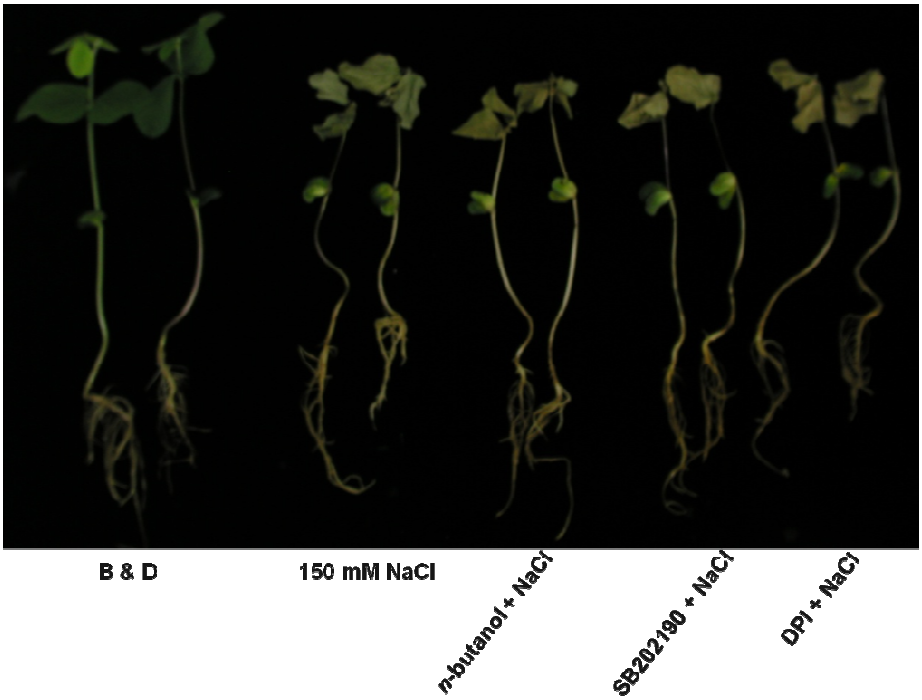


Figure 2-14. Phenotype of 150 mM NaCl and various inhibitors co-treated soybean seedlings. After soybean seedlings were grown in B & D solution for 10-days, the seedlings were more cultivated in 150 mM NaCl, 1% *n*-butanol + 150 mM NaCl, 30 μM SB202190 + 150 mM NaCl and 50 μM DPI + 150 mM NaCl for 5-days, respectively. B&D solution treated soybean seedlings were used as a control.

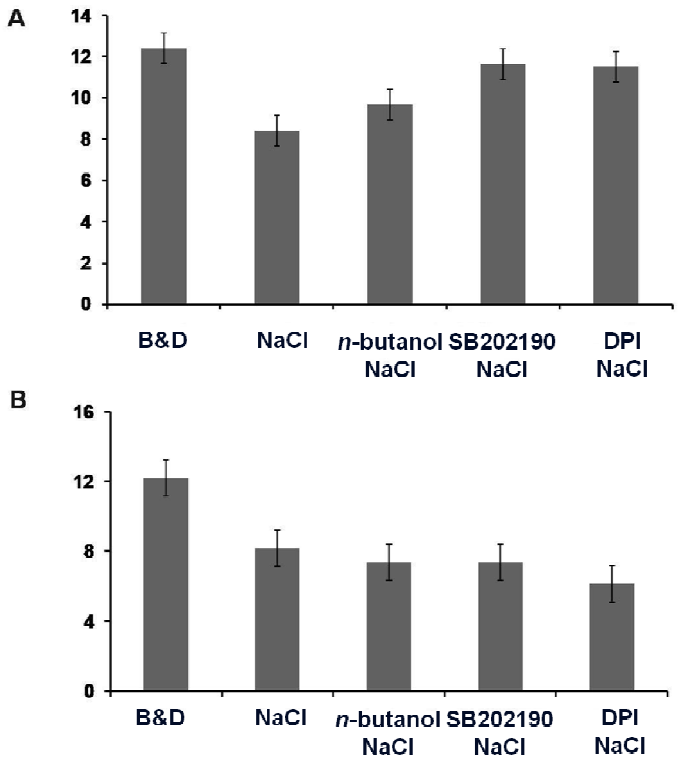


Figure 2-15. Root and shoot length in soybean seedlings grown under 150 mM NaCl and various inhibitors co-treatment. 10-days old soybean seedlings were treated with 150 mM NaCl and inhibitor for 5-days and measured root length (A) and shoot length (B). B&D solution treated soybean seedlings were used as a control. Mean ± SD of 5 samples.

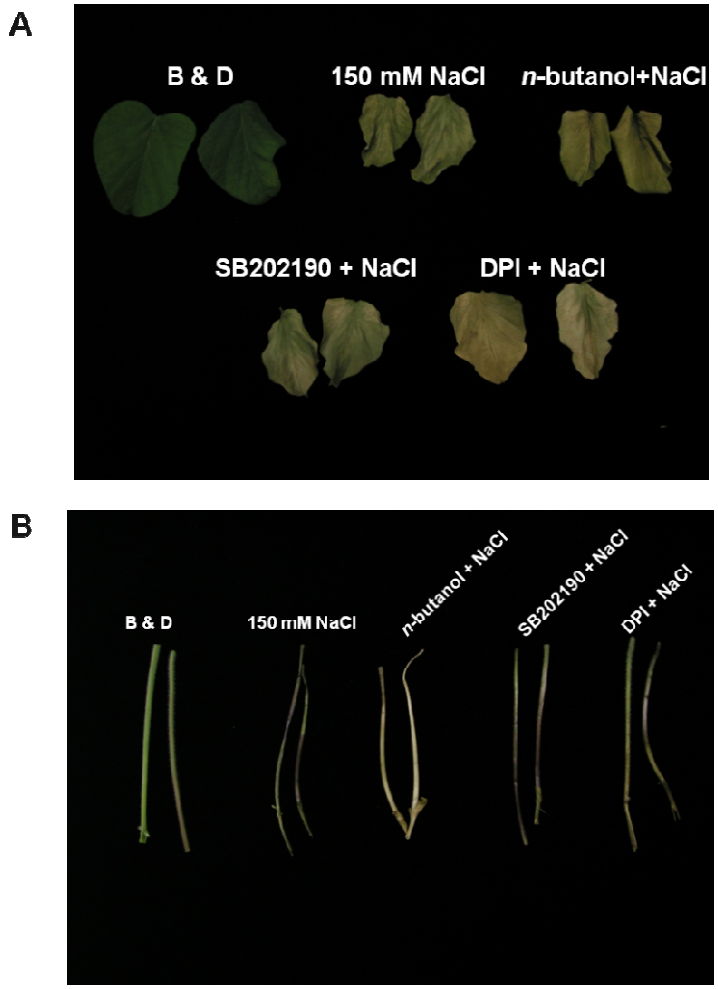


Figure 2-16. The color of leaf and stem in 150 mM NaCl and various inhibitors co-treated soybean. 10-days old soybean seedlings were treated with 150 mM NaCl and inhibitor for 5-days and checked leaves (A) and stem (B) colors. B&D solution treated soybean seedlings were used as a control.

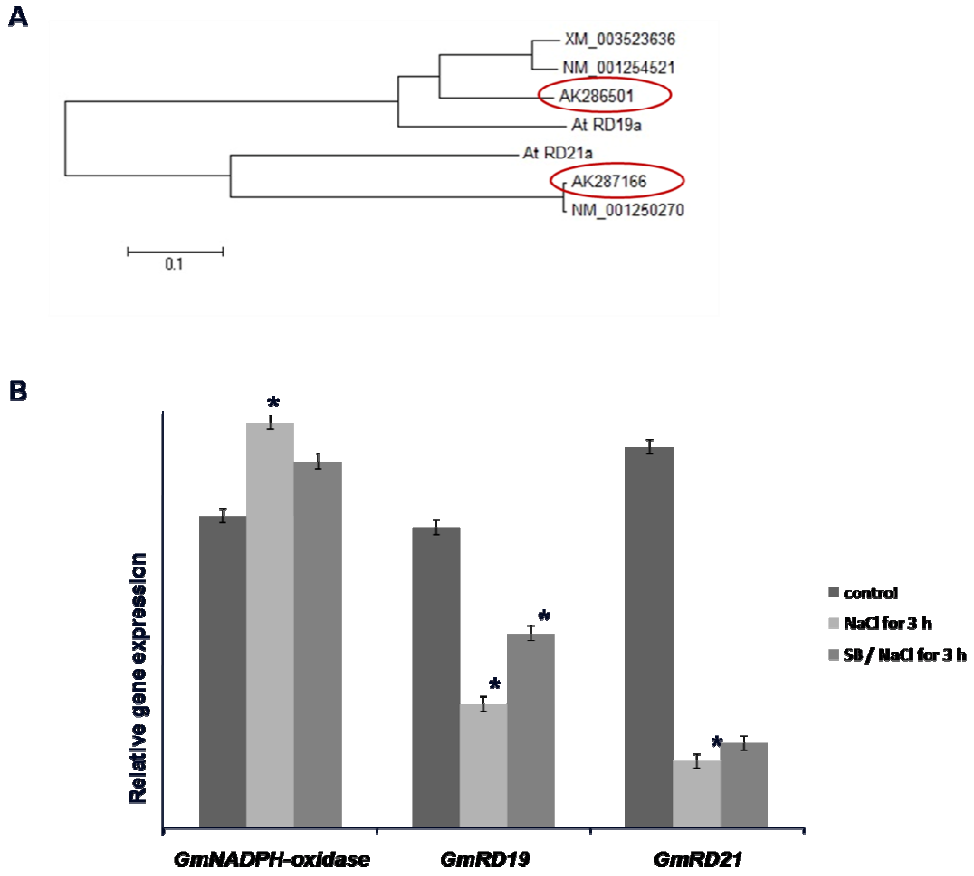


Figure 2-17. Phylogenetic tree and RT-PCR of salt stress-related soybean genes in 300 mM NaCl treatment. A. Phylogenetic tree of *GmRD19* (AK286501) and *GmRD21* (AK287166) with *Arabidopsis RD19* and *RD21*. B. RT-PCR of *GmNADPH-oxidase*, *GmRD19* and *GmRD21* under 300 mM NaCl treatment for 3 h and, 30 μM SB202190 treatment for 1 h after 300 mM NaCl treatment for 3 h. Mean ± SD of 3 samples. (* significant at $P < 0.01$)

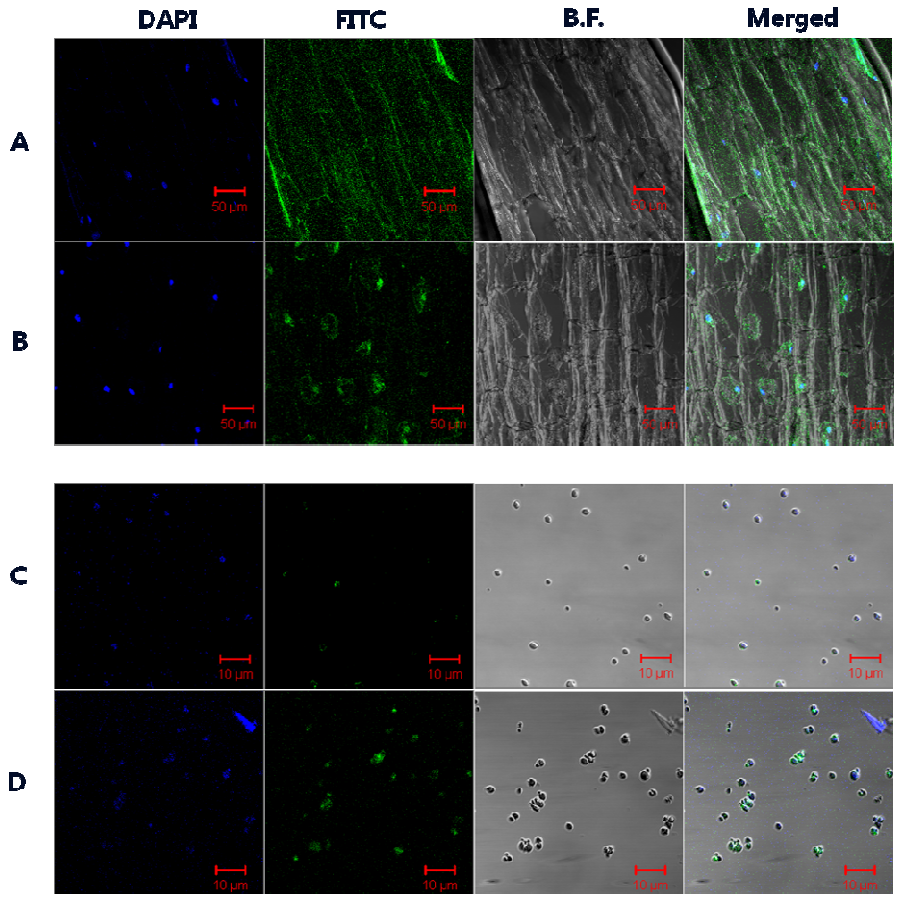


Figure 2-18. Immunolocalization of GMK1 in NaCl treatment. A, Longitudinally cut soybean root was treated with anti-GMK1 serum, and then treated with FITC-conjugated antibody. B, Soybean seedling treated with 300 mM NaCl for 60 min was longitudinally cut and was treated with anti-GMK1 serum and FITC-conjugated antibody. C, Nuclei were isolated with PARTEC (Münster, Germany) nuclear isolation reagents and then treated with anti-GMK1 antibody and FITC-conjugated antibody. D, Nuclei were isolated from 300 mM NaCl treated soybean seedlings and were handled in the same manner as C. All of these images were obtained from confocal microscopy. Nucleus was detected as blue color by DAPI staining.

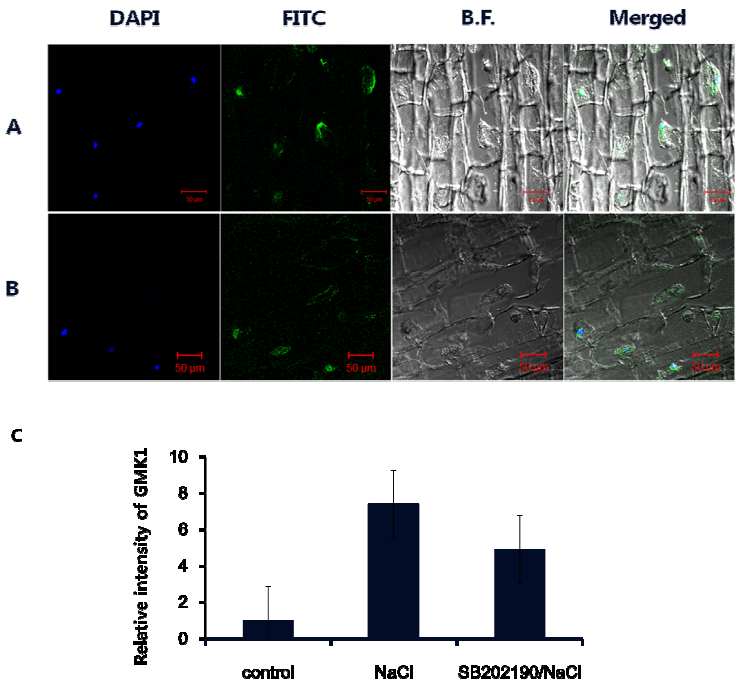


Figure 2-19. Translocation of GMK1 was disrupted by SB202190 under salt stress. A, Soybean seedlings were treated with 300 mM NaCl for 60 min and the seedlings were cut with longitudinally. The samples were treated with anti-GMK1 and FITC-conjugated antibody. B, Soybean seedlings were treated with SB202190 for 60 min before treatment with 300 mM NaCl for 60 min. The seedlings were handled with same manner descried at A. All of the images were obtained by confocal microscopy (LSM-510, Carl Zeiss) with Z-stack. DAPI signal is shown as a blue. C, nuclei were isolated from indicated samples and treated with anti-GMK1 and FITC-conjugated antibody. GMK1 signal was detected from the samples with confocal microscopy and the signal intensities were measured by Image J program. GMK1 intensity of control seedlings nuclei was set as a 1. control, D.W. treated seedlings; NaCl, seedlings treated with 300 mM NaCl for 60 min; SB202190/NaCl, soybean seedlings were treated with 30 μM SB202190 for 60 min and then treated with 300 mM NaCl for 60 min. Mean ± SD of 2 samples.

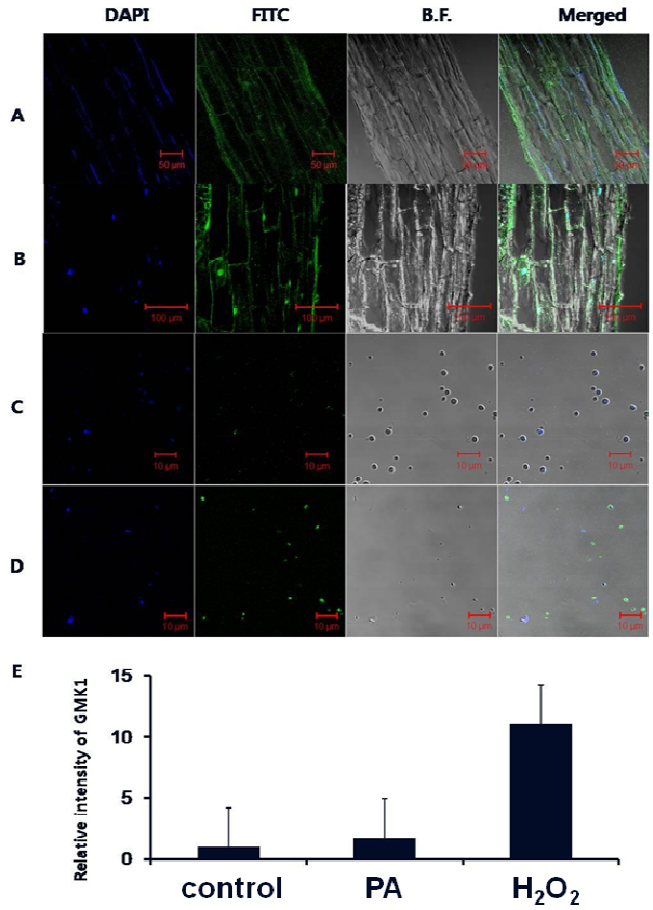


Figure 2-20. Immunolocalization of GMK1 under PA and H_2O_2 treatment. A, Soybean seedlings were treated with 50 μM PA for 60 min and longitudinally sectioned. The samples were treated with anti-GMK1 antibody and FITC-conjugated antibody. B, soybean seedlings were treated with 1 mM H_2O_2 and handled with same manner of A. C and D, Nuclei were isolated from sample A and B, respectively, and then treated with anti-GMK1 and secondary antibody. DAPI signals were detected as blue indicating nuclei. All of the images were obtained by confocal microscopy (LSM-510, Carl Zeiss). E, GMK1 signal was measured by Image J program from nuclei of C and D with control. Control intensity was set as a 1. Mean ± SD of 3 samples.

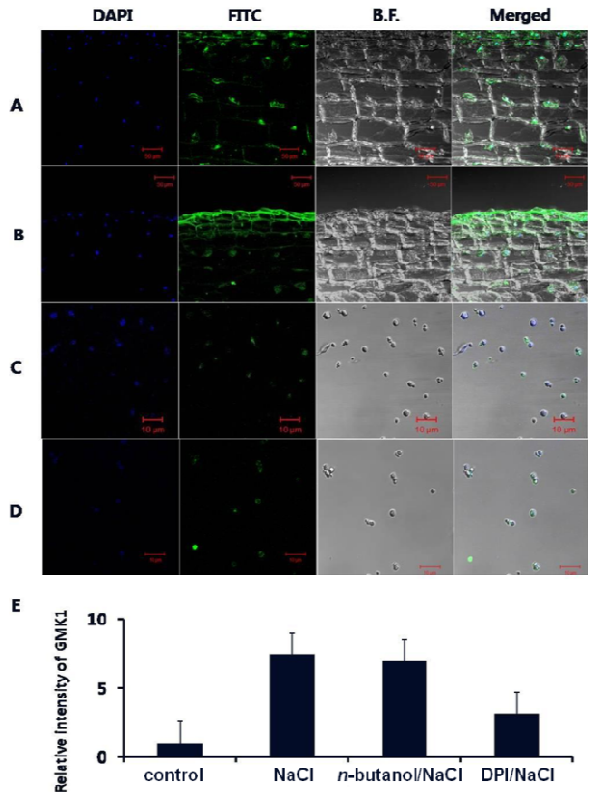


Figure 2-21. Translocation of GMK1 is not disturbed by *n*-butanol but reduced by DPI. A, Soybean seedlings were treated with 1% *n*-butanol for 60 min before the treatment with 300 mM NaCl for 60 min. The seedlings were cut longitudinally and treated with anti-GMK1 antibody and FITC-conjugated antibody. B, Soybean seedlings were treated with 50 μM DPI for 60 min and treated with 300 mM NaCl for 60 min. The seedlings were handled with same manner of A. C and D, nuclei were isolated from sample A and B, respectively and then treated with anti-GMK1 antibody and FITC-conjugated antibody. All of these images were obtained by confocal microscopy (LSM 510, Carl Zeiss). Nucleus was shown as blue signal by DAPI staining. E, GMK1 signal intensity was measured by Image J program from nuclei of C and D with control and NaCl treated seedlings. Control intensity was set as a 1. Mean ± SD of 3 samples.

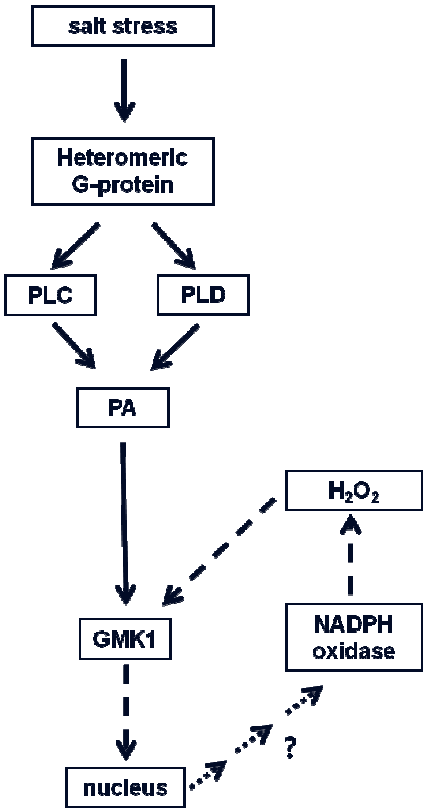


Figure 2-22. A schematic model of the salt stress signaling pathway in soybean. Salt stress could activate heterotrimeric G-protein, and activated heterotrimeric G-protein could deliver the signal to PLC and PLD. PLC makes IP_3 and DAG from PI. DAG is changed to PA by DAG kinase. PLD hydrolyses PC, producing choline and PA. Therefore, PA is made from two phospholipases. PA activates GMK1 (→) and hydrogen peroxide generated from NADPH-oxidase also activates GMK1 in later stage of salt stress and mediates nuclear translocation of GMK1 (- →). NADPH-oxidase is regulated by nuclear translocated-GMK1 with indirectly (-->). **DAG**, diacylglycerol; **IP_3**, inositol 1, 4, 5-phosphate; **PA**, phosphatidic acid.

2-4. DISCUSSION

Several studies on MAPKs in plants have been reported. However, the functional roles of MAPKs in salt stress signaling are largely unknown. In addition, a salt stress-activated MAPK in soybean has yet to be reported yet. This study reports the identification of a salt stress-activated soybean MAPK, GMK1. Upon treatment with NaCl, kinase activity was detected in only one protein band, and this protein was confirmed to be GMK1 (Fig. 2-1A and B). Daxberger et al. (2007) cloned six soybean MAPKs, and we also cloned them using the public soybean EST database. One of these MAPKs, GMK1, orthologous to SIMK, has the same sequence and epitope of GmMPK6. Moreover, a 49-kD wound-induced soybean MAPK is also immunoprecipitated by the anti-SIMK antibody (Lee et al., 2008). It has the same epitope as GMK1. Taken together, these data provide evidence that GMK1, like AtMPK6, is activated by various biotic and abiotic stresses.

In tobacco, WIPK and SIPK are activated by wounding; however, these two MAPKs are regulated differently (Zhang and Klessig, 2000). Therefore, investigation of the mechanism of GMK1 regulation in salt stress is needed. The RNA transcript and protein levels of GMK1 were not changed, but its activity was increased in a concentration-dependent manner, and modulated in a time-dependent manner (Fig. 2-1 and 2-2). The activity of GMK1, like that of SIPK, AtMPK6, and SIMK, was regulated by post-translational modification under salt stress.

Many salt stress signaling are similar with osmotic stress because salt stress induces osmotic stress on plants. 47 kD soybean MAPK was activated by 300 mM NaCl and 300 mM mannitol treatment (Fig. 2-3). However, the activity was different following treatment. This result means that 47 kD soybean MAPK was activated by salt and mannitol, but the activation mechanism is different. AtMPK6 is activated by salt and mannitol, and it is also differently activated in salt and osmotic stress, respectively (Ichimura et al., 2000).

In soybean, the MAPK signaling pathway is poorly elucidated. Moreover, signaling-related studies have been performed in suspension-cultured cells (Lee et

al., 2001; Taylor et al., 2001). To better understand the GMK1 signaling pathway, mastoparan was used. Mastoparan is a wasp venom toxin that activates the Gα subunit of heterotrimeric G-protein (Higashijima et al., 1988). In plants, mastoparan activates calcium spiking analogous to Nod factor-induced responses in *Medicago truncatula* root hair cells (Sun et al., 2007), and activates AtMAPK6 in *Arabidopsis* (Miles et al., 2004) and PLD in tobacco (Dhonukshe et al., 2003). As shown in Fig. 1-3A and B, a MAPK corresponding to GMK1 was activated by 5 μM mastoparan, suggesting that heterotrimeric G-protein has an effect on GMK1 activity in soybean.

Recent studies suggest that multiple phospholipases are involved in the salt stress response and that AtMPK6 activity is mediated by PA (Bargmann et al., 2009; Yu et al., 2010). Therefore, the relationship between PA and MAPK is very important. However, the detailed relationships among heteromeric G-protein, PLD, PLC, and MAPK have not been studied. As shown in Fig. 2-4, GMK1 activity, induced by mastoparan and salt, was diminished by *n*-butanol, neomycin, and suramin, which inhibit PLD, PLC, and heteromeric G-protein, respectively. These data suggest that the salt stress signaling is transduced by PLC and PLD to GMK1. Moreover, GMK1 activity was induced as early as 5 min after 300 mM NaCl treatment (Fig. 2-4D and E), and endogenous PA peaked at that time as well (Fig. 2-6A). These results suggest that heteromeric G-protein activates GMK1 via PA produced by two phospholipases in the early stages of the activation of salt stress responses.

In 300 mM NaCl treatment, GMK1 activity is sustained for up to 30 min (Fig. 2-2A). Therefore, a second activator may regulate GMK1 activity during salt stress. ROS was a good candidate as a regulator, because it is generated during many types of stresses and is a well-known MAPK activator (Yamamizo et al., 2006; Zhang et al., 2006).

To test this possibility, following experiments were carried out. First, ROS generation was investigated using DCFDA and XTT for H_2O_2 and O_2^-, respectively. Normal soybean roots appear green when observed using the FITC channel of a

fluorescent microscope because of their high flavonoid content. However, DCFDA-treated soybean root appears red upon contact with H_2O_2 when observed using the FITC channel. Superoxide was detected using XTT, which is converted to formazan when in contact with O_2^- and the concentration was determined using a spectrophotometer. As shown in Fig. 2-7, ROS generation increased in a time-dependent manner. This result is similar to those of other studies examining soybeans. H_2O_2 is generated in soybean leaves following 150 mM NaCl treatment (Balestrasse et al., 2008) and in drought conditions (Lee et al., 2010). Moreover, it was also reported that ROS is generated by NADPH-oxidase during salt stress (An et al., 2007; Levine et al., 2007). NADPH-oxidase, which is located in the plasma membrane, generates a reactive oxygen intermediate (ROI) under biotic and abiotic stress (Torres and Dangl, 2005; Yang et al., 2007). ROI, like O_2^-, is directly converted to H_2O_2 by superoxide dismutase (SOD). These information suggest that NADPH-oxidase is responsible for this phenomenon because DPI-treated soybeans produced significantly less H_2O_2 than control cells (Fig. 2-8) and SOD activity did not change during salt stress (Fig. 2-12).

Next, to identify whether GMK1 is activated by ROS, seedlings were treated with 1 mM H_2O_2. As shown in Fig. 2-9A and B, GMK1 was activated as early as 3 min after treatment and its activity was sustained for up to 60 min. Moreover, GMK1 activity was not reduced by PA generation inhibitors (Fig. 2-9C), suggesting that H_2O_2 regulates GMK1 activity independently of PA though PLD and PLC regulation.

From these results, ROS was thought to be a late stage activator for GMK1 during salt stress. To test this hypothesis, soybean seedlings were treated with DPI followed by treatment with 300 mM NaCl at different time points. In ROS generation-blocked seedlings, GMK1 activity was rapidly decreased beginning 10 min after treatment, while relatively strong activity was observed at 5 min (Fig. 2-10A). These data suggest that ROS is involved in sustained GMK1 activity from 10 min after salt treatment.

The effect of inhibitors against PA generation was examined. Neomycin and *n*-butanol significantly diminished GMK1 activity 3 and 5 min after 300 mM NaCl treatment, respectively (Fig. 2-10C and D). Moreover, PA levels peaked at 5 min of 300 mM NaCl treatment (Fig. 2-6). These data also strongly support the hypothesis. Thus, salt stress-activated GMK1 is dually regulated by two signal molecules, PA and H_2O_2, at different time points. PLC activation occurred within several seconds, whereas PLD activation required longer (minutes) than PLC in mammalian cells (Nishizuka, 1995). This study supports our data.

Although our data clearly showed that both PA and H_2O_2 regulate GMK1 activity, the relationship between PA/GMK1 and H_2O_2 production has not been well established. In tobacco, MAPK signaling regulates the NO- and NADPH-oxidase dependent oxidative burst (Asai et al., 2008), and positive feedback regulation between MAPK and ROS have been reported (Yamamizo et al., 2006; Zhang et al., 2006). However, AtMPK9 negatively regulates ROS accumulation during mechanical wounding (Takahashi et al., 2011). This information suggests that regulation of ROS generation by MAPK is different depending on the conditions. Therefore, I also investigated the relationship between GMK1 and ROS generation. The MAPK inhibitor SB202190 reduced ROS generation as well as GMK1 activity in NaCl treatment (Fig. 2-11A and B). Moreover, O_2^- generation in protoplasts containing a constitutively activated form of GMKK1 was increased compared to the vector control (Fig. 2-11C and D). These results may suggest that GMK1 also regulates ROS generation.

However, the time points of regulation in ROS generation by PA and GMK1 differ. ROS generation was reduced 30 min after NaCl treatment in *n*-butanol- or neomycin-treated soybeans in which PA generation was inhibited (Fig.2-13). However, ROS significantly increased even after 30 min of 300 mM NaCl treatment (Fig. 2-7A). Therefore, PA can directly regulate ROS generation during salt stress. Previous studies also have shown that PA binds directly to NADPH-oxidase (Zhang et al., 2009). However, in SB202190-treated soybeans in which

activation of GMK1 had been inhibited, ROS generation was reduced compared to the control only 120 min after NaCl treatment (Fig. 2-11B). To further examine these seemingly contradictory data, I introduced TESD-GMKK1 to protoplast. After 10 h of gene expression, protoplast was treated with XTT for 2 h and acquired images under a bright field microscope to observe the conversion of XTT to a red color by O_2^-. As shown Fig. 2-11C, more O_2^- was generated than in the vector control, but not significantly. However, after an additional 8 h of incubation with XTT, ROS generation increased by more than 3-fold (Fig. 2-11D), suggesting that GMK1 indirectly regulates ROS generation during salt stress.

Two types of NADPH-oxidases, StrbohA and StrbohB regulate ROS generation with different time points of elicitor treatment in potato (Yoshioka et al., 2001). StrbohA is involved in ROS generation within 1 h of elicitor treatment, while elicitor-induced StrbohB is involved after 6 h of the treatment. In soybean genome, multiple NADPH-oxidase related genes are exist (www.phytozome.net) and GMK1 is translcoated to nucleus by NaCl treatment. Therefore, GMK1 could regulate gene expression of other type of NADPH-oxidase in soybean.

Salt stress affects growth inhibition, metabolic toxicity and disruption photosynthesis to plant. In soybean, salty environment makes plant mortality, leaf necrosis, and accumulation of chloride in stems and leaves, and reduced green leaf color (Wang and Shannon, 1999). These morphological effects of salt stress are also appeared in this experiment. The growths of 150 mM NaCl treated soybeans were reduced than normal seedlings as well as leaves and stems colors were etiolated (Fig. 2-14, Fig. 2-16). Most of GMK1 signaling pathway related inhibitors were acted as negative effectors to soybean morphology in salt stress, but length of roots are longer than only NaCl treated seedlings (Fig. 2-15). DPI made 150 mM NaCl treated soybean leaves color more severely etiolated, and *n*-butanol also made more etiolated stem color than other inhibitors (Fig. 2-16). However, DPI does not seem to be act in stem; *n*-butanol also could not show the effect on leaves morphology. These results suggested that NADPH-oxidase and PLD have organ specificity to

regulate chlorophyll in salt stress.

MAPK is a functional signaling mediator, regulates various transcription factors in plants (Popescu et al., 2008). Therefore, GMK1 also could regulate various gene expressions in salt stress. To identify the regulation, expressions of *GmNADPH-oxidase*, *GmRD19* and *GmRD21* were analyzed in salt and SB202190 treatment. These genes were changed in high salt stress, and the expressions were also changed by SB202190 treatment (Fig. 2-17B). This result suggested that GMK1 could involve in various gene expression in salt stress.

MAPK signaling pathway transfers signal from out of cell area to nucleus. To examine GMK1 translocation during salt stress, soybean seedlings were treated with 300 mM NaCl for various time; 10, 30, and 60 min. The GMK1 signal did not correlated with the DAPI signal until 30 min after treatment (data not shown). However, after 60 min, the signal corresponded to that of DAPI (Fig. 2-18B). Moreover, GMK1 was only detected in the nuclei of root cells treated with NaCl for 60 min (Fig. 2-18C and D). These results suggest that GMK1 is translocated to the nucleus during salt stress. Localization of MAPK to the nucleus has been previously reported in several stresses (Coronado et al., 2002; Samaj et al., 2002; Liu et al., 2011). However, this study is the first report of translocation of MAPK under salt stress.

Nuclear translocation of MAPK is affected by its activity. Therefore, I tested relationship between activity and nuclear translocation of GMK in salt stress. To reduce GMK1 activity, SB202190 was used to block GMK1 activiy. Down regulation of GMK1 activity by SB202190 is already shown in part 1 study (Fig. 2-11). Nuclear translocation of GMK1 in SB202190-treated seedlings was compared with that in 300 mM NaCl treated seedlings. Translocation of GMK1 in SB202190 treated seedlings compared to control seedlings (Fig. 2-19). This result suggested that nuclear translocation of GMK1 is affected by its activity in salt stress. In mammalian cells, SB202190 inhibits p38 MAPK (Ren et al., 2007) and nuclear translocation of ERK also disturbed by PD98059, MEK inhibitor (Li et al., 2005).

These reports also support my experimental data.

Then, I investigated GMK1 translocation in PA or H_2O_2 treatment, because these two molecules are activator of GMK1 in salt stress. GMK1 was strongly translocated by H_2O_2 treatment but not by PA (Fig. 2-20A and B). This phenomenon also shown in isolated nuclei from the seedlings. GMK1 signal was strongly detected in nuclei of H_2O_2-treated seedlings but not in PA treated-seedlings (Fig. 2-20C and D). Moreover, GMK1 was translocated to nucleus in *n*-butanol-treated seedlings, but its translocation was disturbed in DPI-treated seedlings in 300 mM NaCl treatment (Fig. 2-21). These results suggested that nuclear translocation of GMK1 is mediated by H_2O_2 in salt stress. Consequently, activity of GMK1 is dually regulated by PA and H_2O_2, but translocation is only mediated by H_2O_2.

In mammalian cells, H_2O_2 not only activates p38 MAPK and ERK, but also translocates them to nucleus (Ballard-Croft et al., 2008). PA also activates ERK in various cell types (Kraft et al., 2008), but role of nuclear translocation is not well-characterized. Therefore, it is possible that roles of these two molecules are different in regulation and translocation of GMK1.

NADPH-oxidase activator 1 is phosphorylated by ERK and p38 MAPK in mammalian cells (Lemarie et al., 2008; Oh et al., 2010). In plant, a possibility of SIMK and microtubule interaction is shown in alfalfa (Samaj et al., 2004). These information means that MAPK has role in cytoplasm. PA translocates Raf-1 (MAPKKK) from cytoplasm to plasma membrane (Watanabe et al., 2004), and PA level is only increased at 5 min of salt stress (Fig.2-6). GMK1 is not detected in nucleus at that time. Therefore, GMK1 could also be activated near plasma membrane by PA and may regulate other protein before translocation. Consequently, PA regulates GMK1 at cytoplasm, while H_2O_2 mediates translocation as well as regulation of GMK1.

Many transcription factors are phosphorylated by MAPKs in mammalian cells. In plant, 570 MAPK substrates were detected by protein microarray and most of them were also revealed as a transcription factor (Popescu et al., 2009). To

identify target protein of GMK1 in salt stress, total protein was extracted from 300 mM NaCl treated seedlings and immunoprecipitated with anti GMK1 antibody and analyzed co-immunoprecipitated proteins (data not shown). Among them, TF2B were detected, repeatedly. Therefore, TF2B could be GMK1 target protein.

TF2B is a general transcription factor, makes up RNA polymerase 2 pre-initiation complex. RNA polymerase not recognize transcription initiation site in TF2B mutant (Lee and Young, 2000). This information suggests that GMK1 has possibility to regulate gene expression.

Based on the results, this study proposes a signaling pathway involving GMK1 during salt stress. Salt stress increases PA for 5 min in the presence of PLD and PLC; PA induces activity of GMK1 and NADPH-oxidase. Next, O_2^- generated from NADPH-oxidase is directly converted to H_2O_2 by SOD, and H_2O_2 maintains the activity of GMK1 at later times. Activated GMK1 is translocated to nucleus and may induce downstream reactions including gene expression of NADPH-oxidase (Fig. 2-22).

Even this study suggests GMK1 signaling pathway in salt stress, several points are unclear. First, OXI1 is a serine/threonine protein kinase activated by H_2O_2 and PA in *Arabidopsis*. The OXI1 null mutant is unable to activate AtMPK3 and AtMPK6 following H_2O_2 treatment (Rentel et al., 2004), suggesting that OXI1 functions downstream of ROS but upstream of the MAPK module (Pitzschke and Hirt, 2006). However, OMTK1 is a MAPKKK of alfalfa that is directly activated by H_2O_2 (Nakagami et al., 2004) and PA generated from phospholipase Dα directly activates AtMPK6 during salt stress (Yu et al., 2010). Further studies are necessary to understand the functions of the upstream elements in the GMK1 signaling pathway.

Second, SB202190 has been widely used as a p38 MAPK-specific inhibitor, but it cannot inhibit extracellular signal-related kinase (Owerkowicz et al.) activity in animal cells. Plant MAPKs include one type of ERK; GMK1 also belongs to this family. However, SB202190 reduces the activity of a

Bradyrhizobium-activated orthologus of SIMK (Fernandez-Pascual et al., 2006), and our data also showed same result (Fig. 2-11C). It may differently regulate MAPK activity in plant.

Third, the ROS generation level increased until 60 min after NaCl treatment, but GMK1 activity decreased after 30 min according to our previous study. There are two plausible explanations for this observation. First, a negative regulator of GMK1 may be present. In *Arabidopsis*, AtKP1 down-regulates AtMPK6 activity (Ulm et al., 2002) and AtDsPTP1 inactivates AtMAPK3/6 (Gupta et al., 1998). Second, GMK1 may be physically isolated from upstream activators such as GMKK1 because GMK1 is translocated from the nearby plasma membrane to the nucleus during salt stress.

Further studies such as the role of PLD and PLC subtypes using loss-of-function mutants are necessary to elucidate the GMK1 signaling pathway during salt stress, since most of data of this study are based on pharmacological evidence.

Chapter 3

Two Soybean Mitogen-Activated Protein Kinase are Involved in *Bradyrhizobium*-Soybean Interaction.

3-1. Introduction

3-1.1. Nitrogen fixation

Nitrogen is a main source of proteins and nitrogen-contained compounds as well as nucleic acids. Approximately 80% of atmosphere consists of N_2. Nevertheless, most of organisms could not use the N_2 because N_2 tightly combined between N atoms with triple-bond. Therefore, most of organisms obtained nitrogen source from ammonia (NH_3) fixed by rhizobia.

Nitrogen fixation is one of main way supplying nitrogen to living organisms, occurred in nodule of legume plant. Root nodulation is started from rhizobia-legume interaction. Legume plants secrete a lot of flavonoid. The flavonoids attract *Rhizobium* to legume root as well as induce generation of Nodulation factor (Nod factor). Nod factor makes root hair cell curling and the bacteria surrounding. Therefore, the bacteria entered to root hair cell. *Rhizobium* moves to cortical cell via infection thread and product NH_3^+ with division and differentiation. Infected root generates symbiosome-contained special organ, nodule from cortical cells.

3-1.2. Nod factor signaling

One of main Nod factor signaling pathway, DMIs signaling is well-elucidated in *Medicago truncatula* though mutant screening (Catoira et al., 2000). The DMI proteins, are most important signaling factors, regulate root hair growth. The LRR receptor kinase, DMI2 is located in the plasma membrane whereas DMI1 is located in the nuclear envelope (Ane et al., 2002). Therefore, DMI2 is thought to be upstream of DMI1. All these proteins are required to induce calcium spiking, and are regulated by the calcium/calmodulin dependent protein kinase DMI3 (Olah et al., 2005). Therefore, DMI3 activates the Nod factor response genes and the response is mediated by calcium.

The other signaling is phosphatidic acid (PA) related signaling pathway. Heteromeric G-protein is play an important role in Nod factor signaling as well as

activates two phospholipases, phospholipase C (PLC) and phospholipase D (PLD) (Legendre et al., 1993; Pingret et al., 1998; Dhonukshe et al., 2003). These two phospholipases generate PA from phosphatidylcholine (PC) and Phosphatidylinositol 3-phosphate (PI_3P), respectively. *n*-butanol has been widely used as a phospholipase D (PLD) inhibitor because it combines to PLD with phosphatidylcholine. Neomycin is also a well-known PLC inhibitor that blocks many animal and plant phospholipase C (PLC) activities. In *Arabidopsis*, neomycin reduced activity of PLC, which was activated by cold treatment, but it is no less effective in PLD activity (Ruelland et al., 2002).

PA is involved in various signaling pathways including mitogen activated protein kinase (MAPK) signaling. For examples, PA directly combines and activates AtMPK6 in *Arabidopsis* (Yu et al., 2010) and activates GMK1 in soybean (Lee et al., 2001). However, PA and MAPK interaction in Nod factor signaling is not well-studied.

3-1.3. MAPK

Approximately, 10% of all kinase are related in MAPK signaling pathway in plant (Colcombet and Hirt, 2008) and these signaling are involved in various pathway such as development, biotic and abiotic stresses. MAPK signaling is transferred by phosphorylation. MAPK is activated by upstream kinase, MAPK kinase (MAPKK) also activated by MAPKK kinase (MAPKKK). In soybean, at least 56 MAPKs and 80 MAPKKs and 100 MAPKKKs are encoded at genome (Liu et al., 2011). However, signaling pathway of soybean MAPKs are hardly studied and elucidated.

MAPK activities are known to be regulated in different ways. Activities of SIPK/AtMPK6/SIMK/GMK1 were regulated at the post-translational level, and those of WIPK/AtMPK3/SAMK were regulated at the transcriptional level (Seo et al., 1995; Bogre et al., 1997; Zhang and Klessig, 1997).

In previous study, 47-kD MAPK was revealed as a GMK1 in GCF-

activated two MAPKs. However, 44-kD MAPK was not elucidated. In this part, I identified GCF-activated two MAPKs and regulation mechanism of GMK2 activity in soybean and *Bradyrhizobium* interaction. Activity of GMK2 is regulated at translational level and the activity also regulated by PA and calcium signaling in the interaction.

3-2. Materials and methods

3-2.1. Plant material

Glycine max L. seeds were surface-sterilized with bleach solution (0.2% Chlorox) for 5 min, followed by five washes with sterilized distilled water. Seeds were placed on wet paper towels for germination in a growth chamber (25°C, 60% humidity) for 3 days under dark conditions. The seedlings were transferred to B&D solution (Broughton and Dilworth, 1971) contained tube and grown for 2 weeks and treated GCF for desired time points.

3-2.2. Bacterial culture and preparation of culture filtrate

Bradyrhizobium japonicum strain USDA110 was grown at 28°C in 100 mL of yeast mannitol broth (YEM; Vincent, 1970). Culturing conditions included shaking at 150 rpm for 3 d on an orbital shaker, followed by sub-culturing into 200 mL of a YEM medium. After 5 d (OD600 of 0.4 to 0.6), the *B. japonicum* culture filtrate was prepared as described previously (Ghelue et al., 1997). Briefly, 5 μM genistein was added to bacterial cultures to induce the synthesis and production of Nod factor, and the same volume of DMSO was added to the control reaction. After another 3 d of incubation, the cultures were centrifuged and the supernatants were filter-sterilized with a 0.22 μm syringe filter (Millipore, Billerica, MA, USA). The resulting cell-free culture filtrates were used for our root hair deformation assays and plant treatments.

3-2.3. In-gel kinase assay.

In-gel kinase assay was performed as described previously (Lee et al., 2008). Extracts containing 35 μg of proteins were electrophoresis on 10% SDS-discontinuous polyacrylamide gels embedded with 0.33 mg/ml of myelin basic protein (MBP) in the separating gel as a substrate of MAP kinase. The gels were then run at 80 V in the stacking gel for 1 h and 120 V in the separating gel for 2.5 h. To remove the SDS, the gels were washed three times for 20 min each with 20% propan-2-ol in 50 mM Tris (pH 7.5). The gels were then washed with 50 mM Tris (pH 7.5) 5 mM 2-mercaptoethanol for 30 min to remove the propan-2-ol. The proteins were denatured by incubating for 15 min each in 6, 3, 1.5 and 0.75 M urea in 50 mM Tris (pH 7.5) and 5 mM 2-mercaptoethanol. The proteins were renatured for 24 h at 4°C in six changes of 40 mM Hepes (pH 7.4) 5 mM 2-mercaptoethanol 0.05% Tween 20. The gels were preincubated in 25 ml of 40 mM Hepes (pH 7.4), 10 mM MgCl2, 0.1 mM EGTA and 25 μM ATP for 1 h. The kinase reaction was initiated by the addition of 9.25×10^8 Bq of [γ-^{32}P] ATP and allowed to proceed for 4 h. The reaction was terminated by removal of the kinase buffer and by multiple washed with 5% trichloroacetic acid (TCA) and 1% pyrophosphate. After staining and destaining, the gels were dried into gel dry film and exposed to X-ray film for 7~14 days.

3-2.4. Immunoprecipitaion and in-gel kinae assay

For the immunoprecipitation assay, total protein samples (400 μg) were incubated with anti-GMK1 or anti-GMK2 antibody at 4^{o}C for 2 h, then precipitated with protein A sepharose. After washing with a washing buffer (Lee et al, 2001), the beads were eluted with an SDS sample buffer at 95^{o}C for 3 min and subjected to in-gel kinase.

3-2.5. Total RNA isolation and RT-PCR

400 μg powders of tissue were transferred to clean E-tube and added 1 ml TRIzol (invitrogen). The samples were mixed with 0.2 ml chloroform and

vigorously vortexing for 10 sec. After incubated for 5 min at 4°C, the homogenate was centrifuged for 15 min at 13,000 rpm. Supernatant was transferred to new E-tube and precipitated with equal volume of isopropanol (IPA) for 20 min at -20oC. The precipitated RNA was centrifuged for 20 min at 13,000 rpm at 4oC. The pellet was rinsed with 80% ethanol and centrifuged for additional 10 min at 13,000 rpm. The RNA was resuspended with DEPC-treated ddH_2O. PCR was performed by *GMK2* specific primer for 25 cycles.

3-2.6. Preparation of protein extracts and western blot analysis

Tissue powders were added up to 400 ul in 1.5 ml tube containing protein extraction buffer [50 mM Tris (pH 7.4), 1% NP-40, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM Na_3Vo_4, 1 mM NaF and 1 mg/ml aprotinin, leupeptin, pepstain]. The samples were incubated on ice for 5 min and homogenized for 10 sec by vortexing. After centrifugation at 15,000 rpm for 15 min at 4°C, supernatants were transferred into new tubes. After two times of additional centrifugations, concentrations of protein samples were determined by the Bradford method. Thirty micrograms of total protein were used for SDS-PAGE and transferred onto nitrocellulose membrane (PROTRAN, Germany). The membrane was blocked using blocking solution [5% non-fat dry milk in PBST (137 mM NaCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, 2.7 mM KCl, 0.05% Tween 20)] at room temperature and washed three times with PBST for 30 min. Primary antibody anti-GMK2 [epitop: LNPEYA] was added to the membrane and goat anti-rabbit IgG conjugated to horseradish peroxidase (Promega) was used as a secondary antibody.

3-3. Results

3-3.1. Soybean two MAPKs, GMK1 and GMK2 are activated by GCF treatment

Previous study suggested that two soybean MAPKs are activated by GCF (Genistein induced *B. japonicum* Culture Filtrate) treatment from 30 min to 2 h (Lee

et al., 2008). However, root hair curling by GCF treatment was observed after 12 h of the treatment. To investigate MAPKs activities, soybean seedlings were treated with GCF until 12 h. Two soybean MAPKs were strongly activated at 30 min after GCF treatment. Activity of 44-kD MAPK was disappeared after 3 h of GCF treatment, but GMK1 activity was also gradually reduced after that time (Fig. 3-1).

In soybean, GCF-activated two MAPKs are not completely identified. Only 47-kD MAPK is identified as a GMK1 (Lee et al., 2008). To identify GCF-activated two soybean MAPKs, soybean seedlings were treated with GCF for 30 min and subjected to immunoprecipitation (IP) and in-gel kinase assay, using anti-GMK1 and anti-GMK2 antibodies because orthologus of SIMK and SAMK are activated by *Bradyrhizobium* inoculation in *lupine* (Fernandez-Pascual et al., 2006). GMK1 and GMK2 are also orthologus of these two MAPKs, respectively. As shown Fig. 3-2, GCF-activated two MAPKs were identified as a GMK1 and GMK2.

3-3.2. GMK2 activity is regulated at translational level.

Activity of GMK1 is regulated at post-translational level in GCF treatment (Lee et al., 2008). However, regulation mechanism of GMK2 is not studied yet. To investigate gene and protein expression patterns of GMK2 in GCF treatment, total RNA and protein were isolated from GCF-treated seedlings and subjected to RT-PCR and western blotting analysis. *GMK2* expressions in transcriptional level were not changed for 120 min of GCF treatment (Fig. 3-3A). However, the expressions were changed at translational level. GMK2 protein was barely expressed in normal condition, but rapidly increased at 10 min of GCF treatment. The increase was sustained until 30 min, and reduced the expression after that time (Fig. 3-3B). This result suggested that activity of GMK2 is regulated at translational level.

3-3.3. GMK1 and GMK2 are regulated by PA, while GMK2 is regulated by calcium in GCF treatment.

One of Nod factor signaling is mediated by heteromeric G-protein and

passed to PA generated PLC and PLD (den Hartog et al., 2003). PA directly activates GMK1 in salt stress and wounding (Fig. 2-6; Lee et al., 2001). This information suggests that GMK1 and GMK2 are also regulated by PA in nod factor signaling. In order to elucidate relationship between heteromeric G-protein and MAPKs in GCF treatment, suramin, heteromeric G-protein inhibitor was treated to soybean before GCF treatment for 30 min. In this case, activities of GMK1 and GMK2 were diminished by suramin (Fig. 3-4A).

To identify relationship between PA and GCF-activated two MAPKs, soybean seedlings were treated with PLD and PLC inhibitors, *n*-butanol and neomycin, respectively and then treated with GCF for 30 min. *n*-butanol or neomycin treatment completely reduced activities of two MAPKs activated by GCF (Fig. 3-4B).

PLD generates only PA from PC, but PLC generates IP_3 and DAG from PI_3P. DAG is changed to PA by DAG kinase, and IP_3 directly move to endoplasmic reticulum (ER) and increases cytosol calcium level. Therefore, effects of PLD and PLC are also revealed as effects of PA and calcium. In order to identify the effect of calcium on two MAPK activities in GCF treatment, soybean seedlings were treated with EGTA and $LaCl_3$ before GCF treatment for 30 min. As shown Fig. 3-4C, activity of GMK1 was not affected by EGTA and $LaCl_3$, but that of GMK2 was reduced by the inhibitors. This result suggests that GMK2 is also regulated by intracellular calcium level.

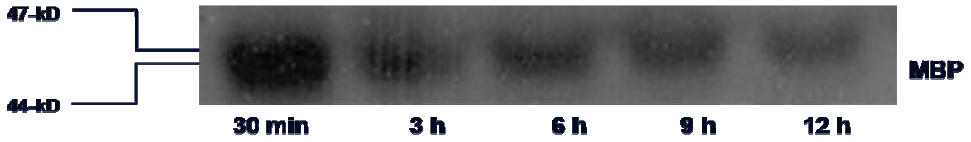


Figure 3-1. In-gel kinase assay of GCF treated soybean seedlings. *Bradyrhizobium* were cultured in YEM for 3-days and were treated with 5 μM genistein, and more cultured for 5-days. Filtrate of the media was treated to 2-weeks old soybean seedlings for indicated time points. Total protein was extracted from the seedlings and was subjected to an in-gel kinase assay.

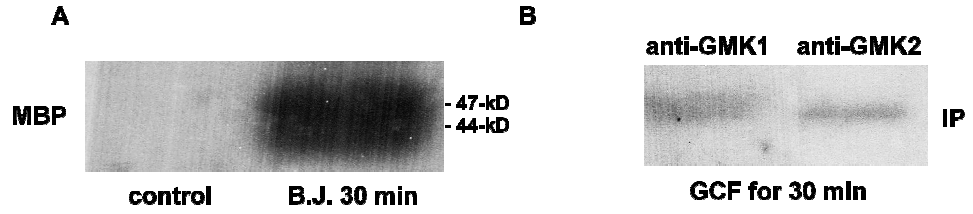


Figure 3-2. Two soybean MAPKs, GMK1 and GMK2, were activated by GCF. A, Total protein was extracted from soybean seedlings treated with GCF for 30 min, and was subjected to an in-gel kinase assay. Uninduced *B. japonicum* Culture Filtrate (UCF)-treated soybean seedlings were used as a control. B, Total protein was extracted from the same samples used in (A) and subjected to immunoprecipitation and an in-gel kinase assay. MBP was used as a MAPK substrate.

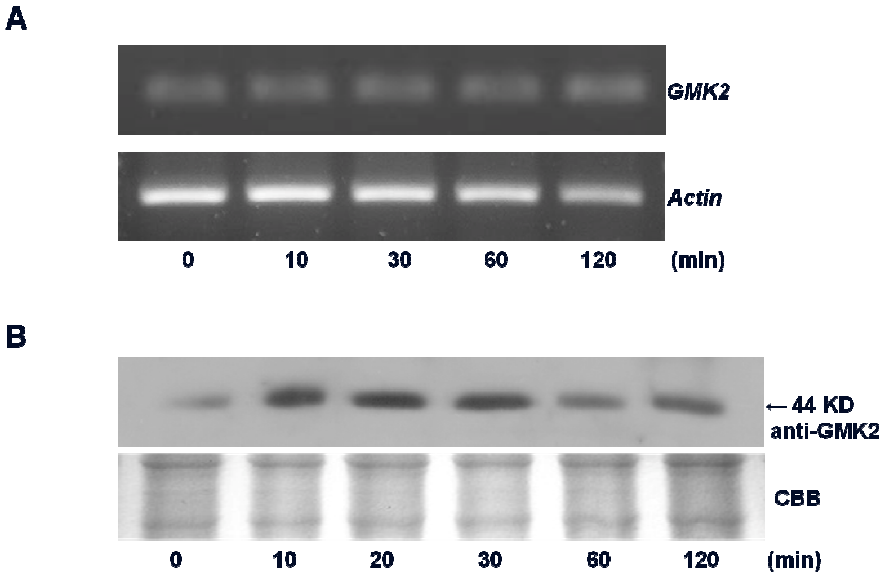


Figure 3-3. Genes and proteins expression of GMK2 in GCF-treated soybean seedlings. A, Soybean seedlings were treated with GCF for indicated time points, and then total RNA was extracted from the seedlings and was subjected to RT-PCR with *GMK2* specific primers. The amounts of RNA were shown with *actin*. B, Total protein was extracted from same samples used in (A) and subjected to immunoblot analysis with GMK2 specific antibody. CBB; coomasie brilliant blue.

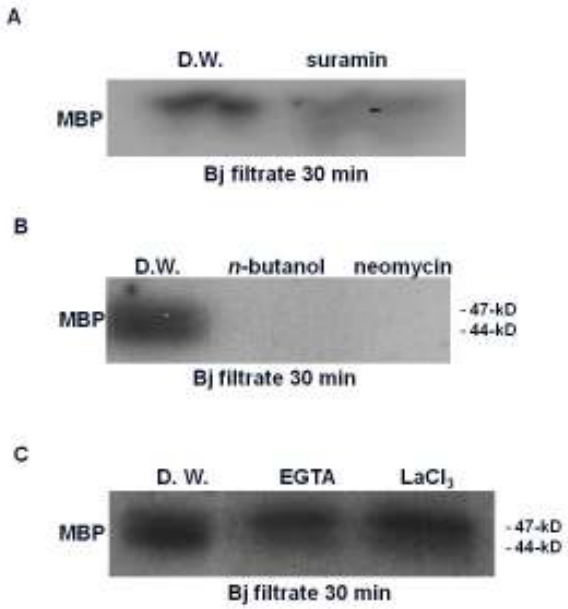


Figure 3-4. The regulation of GCF-activated two MAPKs by suramin, *n*-butanol and neomycin, and effects of calcium signaling inhibitors on the MAPKs. A, Soybean seedlings were treated with 10 μM for 60 min and treated with GCF for 30 min. Total protein was extracted from the seedlings and subjected to an in-gel kinase assay. B**,** Soybean seedlings were treated with 1% *n*-butanol and 15 μM neomycin before treated with GCF for 30 min. Total protein was extracted from these seedlings and was subjected to an in-gel kinase assay. C, After soybean seedlings were treated with 2 mM EGTA and 1 mM $LaCl_3$, the seedlings were treated with GCF for 30 min. In-gel kinase assay was performed with total protein obtained from the seedlings. D.W. was treated as a inhibitors and chemicals control.

3-4. Discussion

Nod factor is a crucial molecule in early stage of *Rhizobium*-legume interaction. It is synthesis and secreted from the bacteria by flavonoids from legume plant. Nod factor is recognized by LRR receptor kinases and transferred the signal to PLD and PLC (Madsen et al., 2003; Radutoiu et al., 2003). However, MAPK involvement in the interaction is not well-studied.

In the previous study, 47- and 44-kD MAPKs were activated by GCF treatment (Lee et al., 2008). This study is the first report of MAPK's involvement in soybean symbiotic interaction. However, the study identified only 47-kD MAPK and investigated MAPKs activities only for 2 h of GCF treatment. Therefore, more elaborated study has been needed. Nod factor-induced root hair curling was occurred after 12 h of bacteria inoculation (data not shown). However, MAPKs activities were not studied until that time. In time-course analysis of MAPKs activities until 12 h of GCF treatment, 47-kD and 44-kD MAPKs were activated at 30 min of the treatment. However, 44-kD MAPK activity was reduced after that time, while 47-kD MAPK was continually activated (Fig. 3-1).

In alfalfa, SIMK regulates root hair tip growth with actin regulation (Samaj et al., 2002) and SIMK and SAMK activities are detected in root hair cells and inhibition of the MAPKs activities also reduced nodule generation in *Lupinus* (Fernandez-Pascual et al., 2006). GCF-activated 47-kD and 44-kD MAPK are orthologus of SIMK and SAMK, respectively, because these two MAPKs were immunoprecipitated by anti-GMK1 and GMK2 antibodies (Fig. 3-2). Therefore, the MAPKs may involve in Nod factor signaling to regulate root hair curling with different time point.

To identify regulation mechanism of GMK2 in GCF treatment, gene and protein expression levels were investigated in the treatment. mRNA level of *GMK2* was not changed during the treatment, but proteins and kinase activities were changed (Fig. 3-3; Lee et al., 2008). This result suggested that GMK2 activity was regulated at translational level. However, many orthologus of GMK2 are regulated

at transcriptional level. Therefore, GMK2 may differently be regulated in symbiotic interaction.

It is well-known that heteromeric G-protein is involved in Nod factor signaling and regulates PLD and PLC (den Hartog et al., 2001). In salt stress, GMK1 is also regulated by PA (Fig. 2-6). However, MAPK signaling pathway in Nod factor signaling is not studied yet. Therefore, it is reasonable to speculate that GCF-activated MAPKs are regulated by PA. GMK1 and GMK2 were completely inhibited by PA generation inhibitors, *n*-butanol and neomycin, respectively (Fig. 3-4B). Moreover, these two MAPKs were also reduced in heteromeric G-protein inhibitor, suramin (Fig. 3-4A). This result suggested that Nod factor signaling from heteromeric G-protein is transferred to GMK1 and GMK2 via PA in soybean.

Moreover, relationship between Ca^{2+} and MAPKs activity in GCF treatment was investigated. Calcium level is rapidly increased by Nod factor in root hair cell, termed calcium spiking (Ehrhardt et al., 1996). Calcium spiking leads to calcium dependent protein kinase activation and many Nod factor signaling (Lemrow et al., 2004) as well as MAPK activation (Kurusu et al., 2005). EGTA and $LaCl_3$ treatment reduced only GMK2 activity in GCF treatment (Fig. 3-4C). This result suggested that activity of GMK2 is regulated by calcium level in Nod factor signaling.

In soybean suspension cultured cells, OGA activates 47-kD and 44-kD MAPKs, but only 44-kD MAPK is regulated by calcium (Taylor et al., 2001). OGA-activated 44-kD MAPK and GMK2 have similar characteristics such as molecular weight and regulation by Ca^{2+}, especially GMK2 is an orthologus of pathogen elicitor-activated AtMPK3. This information shows possibility that OGA-activated 44-kD MAPK is a GMK2.

Even though GMK1 and GMK2 were activated by GCF treatment, it is not clear that whether the two MAPKs are involved in Nod factor signaling. Nod factor-induced root curling is occurred in root hair cells. However, activity was measured from whole seedling's root. On the other hand, AtMPK6 and AtMPK3 were

activated by pathogen elicitor in *Arabidopsis* and GMK1 and GMK2 also are activated by β-glucan elicitor (Ligterink et al., 1997; Daxberger et al., 2007). Therefore, activation of GMK1 and GMK2 may be induced by *Bradyrhizobium japonicum* elicitor. To identify role of GMK1 and GMK2 involvement in symbiotic interaction, more elaborated studies such as activation of MAPK by pure Nod factor and changing root hair deformation or nodulation in RNAi line of MAPK are needed.

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Conclusion

MAPK is activated by various biotic or abiotic stresses. However, the regulation mechanism and signaling pathway of plant MAPK under abiotic stress are largely unknown and role in symbiotic interaction, *Rhizobium*-legume is also hardly known yet.

Salt stress generates phosphatidic acid (PA) and reactive oxygen species (ROS). PA is generated by PLD and PLC has been known to be a MAPK activator. Heteromeric G-proteins are also known to regulate PLD and PLC. However, these relationships on MAPK activity are not well studied. Moreover, several reports suggest ROS, also well-known MAPK activator, is generated from NADPH-oxidase in salt stress. Therefore, this study is investigated signaling pathway of GMK1 (*Glycine max* MAP kinase 1) with these components in salt stress, and obtained following results in part 1:

1) Salt stress induces GMK1 activity, whereas inhibitors against heteromeric G-protein, PLD and PLC inhibit its activity, respectively. PA activated GMK1 and endogenous PA level was increased in 5 min after 300 mM NaCl treatment. These data suggest that GMK1 is regulated by PA via heteromeric G-protein, PLC and PLD in early stage of salt stress.

2) ROS generation was increased with a time-dependent manner, and exogenous H_2O_2 directly activates GMK1 even in the PA generation inhibitors. GMK1 activity was strongly induced and sustained up to 30 min of NaCl treatment. However, its activity was almost disappeared by NADPH-oxidase inhibitor (DPI), except 5 min treatment. Moreover, *n*-butanol and neomycin strongly reduced GMK1 activity in 5 min of NaCl treatment. These data suggest that activity of GMK1 is regulated by H_2O_2 in later stage of salt stress.

3) GMK1 was translocated to nucleus by salt stress. The translocation is reduced by SB202190 and DPI, but not affected by *n*-butanol treatment.

Consequently, I propose that soybean GMK1 is dually regulated by PA and H_2O_2 with different regulation time point and nuclear translocation of GMK1 is mediated by H_2O_2 in the salt stress signaling. To my knowledge, this study is the

first report on the salt stress activated MAPK in soybean and expands current information further on the relationship among PA, ROS and MAPK in plants.

In part 2, effects of Genistein induced *B. japonicum* Culture Filtrate (GCF) on two MAPKs was investigated. In GCF treatment, 47-kD MAPK and 44-kD MAPK are activated. 47-kD MAPK is already identified as a GMK1. That is regulated at post-translational level in the treatment.

On the other hand, 44-kD MAPK was identified as a GMK2. GMK1 was continually activated up to 15 h of treatment, but activity of GMK2 was decreased after 3 h of GCF treatment. Moreover, GMK2 was regulated at translational level. Therefore, roles of these two MAPKs are different in symbiotic interaction. Activity of GMK1 and GMK2 were reduced by suramin and *n*-butanol, but activity of GMK2 was affected by Ca^{2+}. This result suggested that the MAPKs are regulated by heteromeric G-protein and PA signaling.

Even though the GMK1 and GMK2 were activated by GCF, precise role of the MAPK in symbiotic interaction is not evident. Therefore, more elaborated studies are needed.

Taken together, this study showed that MAPKs of soybean, like other plants, are involved in both abiotic stress (salt stress) and biotic stress (*Bradyrhizobium*), and regulated by heteromeric G-protein and PA signaling. Moreover, the study also expands current information of MAPK further. However, to elucidate their role further, studies on the upstream elements, target proteins of them and *in vivo* functional analysis using RNAi transformations are needed. According to the recently published genome project, at least 56 MAPKs are existed in soybean. However, only a few MAPKs are reported up to now.

국문초록

콩 (*Glycine Max* L)에서의 MAPKs에 관한 최초의 연구는 2000년대 초반에 보고되었다. 하지만 이 MAPKs의 규명과 신호전달과정은 거의 알려져 있지 않고 있다. 따라서 본 연구자는 염 스트레스에서 활성화되는 47-KD의 MAPK인 GMK1과 *Bradyrhzobium japonicum* 배양액 여과물의 처리에 의해 활성화되는 두 개의 MAPKs인 GMK1과 GMK2에 관한 연구를 수행하였다. 염 스트레스에서는 GMK1의 활성이 300 mM까지 처리 후 5분에서 염의 농도에 따라서 증가 하였고, 그 조절 기작은 post-translational modification에 의해 조절됨을 확인하였다. 또한 heteromeric G-protein의 활성제인 mastoparan이 GMK1을 활성화하는 것을 확인하였으며 그 활성이 phspholipse D의 억제물질인 *n*-butanol, phspholipase C 억제물질인 neomycin에 의해 감소함을 확인하였다. 더욱이 300 mM에 의한 GMK1의 활성은 heteromeric G-protein 활성 억제 물질인 suramin, *n*-butanol 그리고 neomycin에 의해 각각 그 활성이 감소하였다. 외생적 phosphatidic acid (PA)를 처리했을 때 직접적으로 GMK1이 활성화 되는 것을 확인하였으며 내생적 PA의 생성양은 300 mM의 염 처리 후 5분에서 최고에 이르렀다. 이러한 결과를 바탕으로 염 스트레스의 초기 신호전달 과정은 heteromeric G-protein으로부터 phsopholipase C, phospholipase D를 거쳐 GMK1으로 전달된다는 이론을 제안하였다. 한편, H_2O_2는 PA 생성 억제 물질이 있어도 GMK1을 활성화시킴을 보였다. 하지만 300 mM NaCl처리 시 5분 이 후에서의 GMK1의 활성은 NADPH-oxidase의 활성 억제 물질인 diphenyleneiodonium (DPI)에 의해 그 활성이 감소함을 보였다. 반면, *n*-butanol과 neomycin은 염 처리 후 5분 이내에서만 그 활성을 감소시켰다. 따라서 300 mM의 염 처리에 의한 GMK1의 활성은 H_2O_2에 의해 유지됨을 알 수 있었다. GMK1과 ROS의 생성관계를 보면, 염 처리시 ROS의 생성량은 MAPK 활성 억제 물질인 SB202190에 의해 감소함을 보였다. 더욱이 *in vitro*에서의 GMK1 활성 단백질인 TESD-GMKK1의 원형질체 과다발현에서는 ROS 생성량이 증가하였는데, 이 현상은 염 처리 후 긴 시간 뒤에 관찰되었다. 이러한 데이터들은 GMK1이 간접적으로 ROS 생성을 조절함을 보인다. 또한 GMK1은 염 처리 후 1 시간 이후에 핵으로 이동하는 것이 관찰되었는데, 이러한 이동은 SB202190에 의해 감소하였다. H_2O_2 처리에 의해 GMK1은 핵으로 이동되는 것이 관찰되었고 염 스트레스에 의한 핵으로의 이동이 DPI의 의해 감소됨이 보였다. 따라서 염 스트레스에서의 GMK1의 핵으로의 이동은 H_2O_2에 의한 것임

을 확인 할 수 있었다.

콩과 *Bradyrhzobium japonicum*과의 관계의 경우, GMK1과 GMK2는 GCF를 15 시간 처리 할 경우 각각 다른 조절 기작을 갖는 것이 보였다. GMK1은 거의 지속적으로 그 활성이 유지되는 것이 비하여 GMK2는 처리 후 3 시간 뒤에 그 활성이 감소됨이 보였다. 더욱이, GMK1은 post-translational modification을 통해 그 활성이 조절되었으나, GMK2의 경우 translational 수준에서 그 활성이 조절되었다. PA 생성 억제 물질의 처리의 경우, GMK1과 GMK2 모두 그 활성이 감소되었으나, 칼슘 신호전달을 막을 경우에는 오직 GMK2만이 그 활성이 감소되었다. 하지만 이러한 결과들이 MAPKs가 Nod factor signaling에 관여한다고 보기에는 부족한 점이 있다. 따라서 rhzobia와의 공생적 관계에서의 MAPK의 역할에 대한 보다 세밀한 연구가 요구된다.

결론적으로, 본 연구는 염 스트레스에서 GMK1이 PA와 H_2O_2에 의해 활성이 조절되며 핵으로의 이동은 H_2O_2에 의해 조절 됨을 밝혔다. 또한, GCF 처리에 의해 활성화 되는 GMK1과 GMK2가 각각 다른 조절 기작에 의해 그 활성이 조절됨을 확인 할 수 있었다.

감사의 글

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