Function and regulation of plant Mitogen-Activated Protein Kinases in metabolic and stress signaling pathways

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Contents

Abbreviations

ABA	Abscisic acid
ACS	1-aminocylcopropane-1-carboxylic acid synthase
ATP	Adenosine triphosphate
AtMKK	*Arabidopsis thaliana* MAPK kinase
AtMPK	*Arabidopsis thaliana* MAP kinase
BSA	bovine serum albumin
CDK	Cyclin-dependent kinase
C.F.U	Colony-forming unit
DAB	3,3-diaminobenzidine
DSP	Dual-specificity phosphatase
DsPTP	Dual-specificity protein tyrosine phosphatase
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
E-FOl	Elicitor preparation of the wilt-inducing fungus *F. oxysporum lycopersici*
eGFP	enhanced green fluorescence protein
EGTA	Ethylene glycol tetraacetic acid
ERK	Extracellular regulated kinase
GUS	β-Glucuronidase
h	Hour
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HR	hypersensitive response
KCl	Potassium chloride
kDa	Kilo-Dalton
LeMPK	*Lycopersicon esculentum* MAP kinase
LpMPK	*Lycopersicon peruvianum* MAP kinase
MAP Kinase	Mitogen activated protein kinase
MAPK Kinase	Mitogen activated protein kinase kinase
MAPKK Kinase	Mitogen activated protein kinase kinase kinase
MBP	Myelin basic protein
MeJA	Methyl jasmonate
MeOH	Methanol
MES	2-(N-morpholino)ethanesulfonic acid
Min	Minute
MKB	MAP kinase-binding
MKP	MAP kinase phosphatase

4-MU	4-Methyl-Umbelliferone
4-MUG	4-Methylumbelliferyl β-D-Glucuronide
$MgCl_2$	Magnesium chloride
NtMEK	Nicotiana tabaum MAPK kinase
OsMAPK	Rice MAP kinase
OX	Overexpression
PGA	Polygalacturonic acid
PGA_1	Prostaglandin A_1
PPA_1	Phytoprostane A_1
PP2A	Protein phosphatases 2A
PR	Pathogenesis related
PTP	Phosphotyrosine phosphatase
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SA	Salicylic acid
SAR	Systemic acquired resistance
Ser	Serine
SIPK	Salicylic acid induced protein kinase
Thr	Threonine
TMV	Tobacco mosaic virus
TPh	Alpha-tocopherol phosphate
Tris	Tris(hydroxymethyl)aminomethane
Tyr	Tyrosine
WIPK	Wounding induced protein kinase
YODA	YDA; MAPKK kinase

Zusammenfassung

In Pflanzenzellen ist die Aktivierung von Mitogen-aktivierten Protein (MAP) Kinasen eine allgemeine Reaktion zur abwehrvermittelten Signaltransduktion. Da die nachgeschalteten Prozesse der Aktivierung der MAP Kinasen in Pflanzen weitestgehend unbekannt sind, wurde die Rolle der MAP Kinasen in Abhängigkeit von stressvermittelnden Stimuli auf die Abwehrmechanismen und den primären Kohlenhydratmetabolismus in Tomate untersucht. Dabei wurde die Beziehung zwischen MAP Kinasen (LpMPK2 und LPMPK3) und der extrazellulären Invertase Lin6, welche ein Schlüsselenzym der apoplastischen Phloementladung darstellt, analysiert. Es konnte gezeigt werden, dass die mRNAs von LpMPK3 und Lin6 sequenziell durch dieselben stressvermittelnden Stimuli (E-Fol, PGA, Verwundung, KCl) induziert werden. Die Induktion des Lin6 Promotors, erkennbar durch eine erhöhte β-Glucuronidase Aktivität 2 Stunden nach Behandlung der Reporterlinien mit Stimuli, war abhängig von der Expression und Aktivierung der LpMPK3. Die vorliegenden Daten zeigen, dass die Induktion von der extrazellulären Invertase Lin6 durch stressvermittelnde Stimuli LpMPK3 bedarf.

Die Behandlung mit Glukose zeigte eine gleichzeitige Induktion der AtMPK4 und AtMPK6 Aktivität, welche durch Anionen-Austausch-Chromatographie separiert und mit Hilfe von spezifischen MAP Kinase Antikörpern nachgewiesen werden konnten. Zusammengefasst lassen diese Daten vermuten, dass die Aktivierung der MAP Kinasen eine zentrale Rolle in der Zucker vermittelten Signalübertragung spielt.

Die Bewegung der Stomata wird durch umweltbedingte Einflüsse wie Lichtintensität, Luftfeuchtigkeit und CO_2-Konzentration kontrolliert. In Arabidopsis wird die Entwicklung und Strukturierung durch eine komplette MAP Kinasen Signalkaskade reguliert. Hingegen ist in höheren Pflanzen wenig über die CO_2 induzierte Signalübertragung bei der Bewegung der Stomata bekannt. Experimente zeigten, dass hohe CO_2 Konzentrationen eine schnelle und kurzzeitige Aktivierung von SIPK und NtMPK4 bewirken. Die Aktivierung der beiden MAP Kinasen könnte bei hoher CO_2 Konzentration die Aktivierung eines Anionenkanals zur Stomata Bewegunng regulieren.

Während in einer Vielzahl von Studien die antioxidativen Eigenschaften von Tocopherolen im Hinblick auf die Regulierung der Stresstoleranz beschrieben ist, sind die nicht-antioxidativen Eigenschaften von Tocopherolen in höheren Pflanzen bis heute nur wenig aufgeklärt. Daher

wurde in Tabak die Funktion von α-Tocopherol auf die Stimuli-induzierte und MAP Kinase-vermittelte Signalübertragung analysiert. Es wurde gezeigt, dass die Aktivierung der MAP Kinase durch die Behandlung mit einem pilzlichen Elizitor und dem Derivat α-Tocopherol-Phosphat induziert wird. Bei der Behandlung mit α-Tocopherol trat dieser Effekt nicht auf. Interessanterweise wurde bei α-Tocopherol im Gegensatz zu Ascorbinsäure ein kurzzeitiger inhibitorischer Effekt auf die Aktivierung der Stimuli-induzierten MAP Kinasen in BY2 Zellen und Tabakpflanzen beobachtet. Der Inhibitor-Aktivitäts-Test ließ vermuten, dass die Applikation indirekt die Aktivität von MAP Kinasen beeinflussen könnte. Diese Ergebnisse deuten auf eine negative Regulierung von α-Tocopherol auf die Stimuli-induzierte Signalübertragung durch Inaktivierung der MAP kinasen hin.

Purin-Analoga sind aufgrund ihrer strukturellen Selektivität als spezifische Proteinkinase-Inhibitoren in Mammalia beschrieben. In dieser Arbeit wurden C2, N6, N9 –trisubstituierte Purine getestet, um grundlegende Beziehungen zwischen chemischer Struktur und inhibitorischen Effekten auf pflanzliche MAP Kinasen zu untersuchen. Die Modifikation der Substitution in der Position C2 und N9 bedingte eine erhöhte inhibitorische Aktivität von 6-(Benzylamino)-Purin Analoga. Daneben lassen die 6-(iso-Pentenylamino)-Purin Analoga vermuten, dass die Addition einer Methylgruppe an der N9 Position verglichen mit der Addition einer Isopropyl-Gruppe eine um das zweifache erhöhte inhibitorische Aktivität bewirkt. Zusammengefasst zeigen die Studien, dass die Selektivität und Wirksamkeit der Inhibitioren durch die Modifikation der chemischen Struktur verbessert wird.

Desweiteren wurde die physiologische Funktion von AtPDP1 (*Arabidopsis thaliana* PLAT domain protein 1) auf die Regulation der Abwehrsignalübertragung, hervorgerufen durch biotsche und abiotische Faktoren, charakterisiert. Interessanterweise bewirkte die Überexpression von AtPDP1 eine erhöhte Empfindlichkeit gegen virulente Pathogene und nekrotrophe Pilze. Zudem begünstigte es die Bildung von Nekrosen aufgrund von unbekannten biotischen Faktoren. Dagegen zeigten diese überexprimierenden Linien während erhöhtem Salzstress eine signifikante Verzögerung der Seneszenz und eine höhere Quantenausbeute des PS II im Vergleich zu den Kontrollpflanzen. Die Ergebnisse weisen sehr deutlich auf eine positive Regulation von AtPDP1 auf die Salztoleranz und erhöhte Empfindlichkeit gegenüber biotischem Stress hin. Daher wird angenommen, dass AtPDP1 durch komplexe Signalwege und Wechselwirkungen während der Stressadaptation reguliert wird.

Summary

Activation of mitogen-activated protein (MAP) kinases is a common reaction of plant cells in defense-related signal transduction pathways. Since the downstream events after the activation of MAP kinases are largely unknown in plants, the role of MAP kinases in the co-ordinate regulation of defense reactions and primary carbon metabolism by stress related stimuli has been analyzed in tomato. Thus, the relationship between mitogen activated protein kinases (LpMPK2 and LpMPK3) and extracellular invertases Lin6, as the key enzyme of an apoplasmic phloem unloading pathway, has been analyzed. The results showed that the mRNAs of LpMPK3 and Lin6 are sequentially induced by the same set of stress related stimuli (E-Fol, PGA, wounding, and KCl). The induction of the Lin6 promotor, as revealed by an increase in β-glucuronidase activity after 2 hours, was dependent both on the expression and activation of LpMPK3. These data suggest that the induction of extracellular invertase Lin6 by stress related stimuli requires LpMPK3.

Glucose, metabolic molecule, was shown to result in the simultaneous induction of AtMPK4 and AtMPK6 activities that could be separated by anion-exchange chromatography, and characterized by differential cross-reaction with MAP kinase antibodies. Taken together, these data suggest that the activation of MAP kinases play central roles in the regulation of sugar signaling.

Stomatal movement is controlled by environmental signals including light intensity, humidity and atmospheric CO_2 level. In *Arabidopsis*, a complete MAP kinase signaling cascade regulates stomatal development and patterning. However, the movement of stomata mediated by CO_2 induced signaling pathways is not fully studied in higher plants. Here, we show that elevated levels of CO_2 induce rapid and transient activation of SIPK and NtMPK4. The activation of both MAP kinases may regulate the anion channel activation for stomatal movement by the elevated level CO_2.

Up to now, the non-antioxidant function of tocopherol is not clear in higher plant, whereas the ability of tocopherol to modulate the stress tolerance mediated by function of antioxidant has been described in numerous studies. Thus, the function of

α-tocopherol in stimuli-induced signal transduction pathways mediated by MAP kinase has been analyzed in tobacco. It has been shown that the activation of MAP kinase was induced by treatment of fungal elicitor and α-tocopherol phosphate but not α-tocopherol. Interestingly, α-tocopherol showed the transient inhibitory effect on the activation of stimuli-induced MAP Kinases in BY2 cells and tobacco plants, whereas ascorbate did not inhibit the activation of MAP kinases. The inhibitory activity test indicated that current application may indirectly affect the activity of MAP kinases. These results suggest that α-tocopherol can negatively regulate stimuli-induced signal transduction pathways via inactivation of MAP kinases.

The purine-analogues have been tested and reported to be specific inhibitors of protein kinases mediated by structural-based selectivity in mammalian. Here, we tested C2, N6, N9-trisubstituted purines to determine basic relationship between their chemical structure and inhibitory activity using a particular plant MAP kinase. The modification of substitution in position C2 and N9 caused the increased inhibitory activity of 6-(benzylamino) purine analogue. In addition, 6-(isopentenylamino) purine analogues suggested that addition of a methyl group to position N9 caused at least 2-fold increased inhibitory activity compared with the addition of isopropyl group. Taken together, our study suggests that the selectivity and potency of inhibitors can be improved by structure modification.

In addition, we have characterized the physiological function of *Arabidopsis thaliana* PLAT domain protein 1 (AtPDP1) in modulating the interaction of defense pathways mediated by biotic and abiotic factors. Interestingly, overexpression of *AtPDP1* resulted in increasing susceptibility of virulent pathogens and necrotrophic fungus, and developing necrosis induced by unknown biotic factors. However, these overexperssion lines showed the significantly delayed senescence and higher level of phosystem II quantum yield compared with control plants against high salt stress. Our results strongly indicate that AtPDP1 positively regulate with salt tolerance, and enhances the sensitivity to biotic stresses. We propose that the *AtPDP1* might be regulated with the complex pathways of interplay among various signaling during stress adaptation.

1. Introduction

Plants have developed an impressive array of defense responses that help minimize or prevent damages caused by a variety of stresses, such as mechanical wounding, UV light exposure and pathogen attack. Some of these defense responses, including ion fluxes and the generation of reactive oxygen species, occur within minutes and may involve events that occur primarily at the post-translational level (Mehdy, 1994; Yahraus *et al*., 1995; Lamb and Dixon, 1997). Protein phosphorylation is the most common post-translational modification, and one of the major mechanisms for controlling intracellular response to extracellular information. In eukaryotic cells, the mitogen activated protein kinase (MAP kinase) cascades, one of the largest and most important categories from protein kinases, are major components of downstream of receptors/sensors that transduce extracellular stimuli into intracellular responses.

1. 1 Activation and Inactivation of MAP kinase cascades

In MAP kinase cascades, MAP kinases form the last component of a "three-kinase" module involving an upstream activator, MAPK kinase (MAPKK), and a MAPKK kinase (MAPKKK) (Figure 1). In response to extracellular stimuli, MAPKK, which is a dual-specificity protein kinase, activates MAP kinase by phosphorylating the Thr and Tyr residues within the TEY or TDY motif, respectively (Kyiakis and Avruch, 1996; Widmann *et al*., 1999; Davis, 2000; Mishra *et al*., 2006). MAP kinases subsequently phosphorylate specific effector proteins, which lead to the activation of cellular responses. At least four MAP kinase cascades have been identified in mammals and six have been detected in yeast (Chang and Karin, 2001). Genetic studies and biochemical approaches have allowed the identification of the associated MAP

kinases and have shown that MAP kinases are not only involved in biotic and abiotic stress responses signaling, but also in cell wall biosynthesis, cell growth, cell differentiation and hormone signaling.

The rapid and transient activation of MAP kinases by extracellular signals is very important for the physiological process regulated by this cascade. However, prolonged or constant activation of MAP kinase cascades can have detrimental consequences for cells (Mansour *et al*., 1994). The constitutively active AtMKK4, AtMKK5, AtMKK7, and AtMKK9 can cause hypersensitive response (HR) in tobacco leaves (Hua *et al*., 2006). Protein phosphatases play a critical role in turning off the activity of MAP kinase cascades through dephosphorylation of threonine and/or tyrosine residues within the activation loop (Luan, 2003).

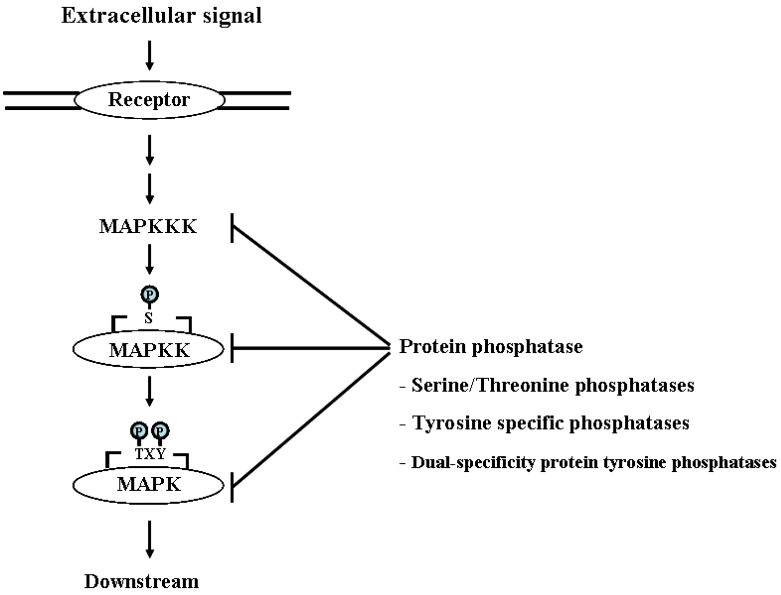


Figure 1. Simplified scheme for MAP kinase activation and inactivation. According to Jonak *et al*., (1999) and Junttila *et al*., (2008) with modifications.

Based on the phosphor-amino acid specificity, protein phosphatases can be divided into serine/threonine phosphatases including PP2A and PP2C, tyrosine specific

phosphatases including STEP, HePTP and PTP-SL, and dual-specificity (tyrosine and serine/threonine; DsPTP) protein tyrosine phosphatases (Gupta *et al*., 1998; Kondoh and Nishida, 2007) including MKPs (MAP kinase phosphatases).

In *Arabidopsis thaliana* genome, seventy-six PP2C-type phosphatase candidates were identified, and divided into ten groups. PP2Cs cooperate with other type of phosphatases in dephosphorylation of the signal transduction cascades including MAP kinase cascades. The function of PP2C has been reported to act as a negative regulator of stress-induced MAP kinase pathways, ABA signaling, and recepotor kinase signaling (Schweighofer *et al*., 2004).

Based on phosphoamino acid specificity, protein tyrosine phosphatases can be divided into tyrosine specific phosphatases and dual-specificity protein tyrosine phosphatases (DsPTPs). They contain a catalytic core motif: (V/I)HCXAGXGR(S/T)G which act as displacing the phosphate group from the substrate and forming a phosphorylcysteinyl intermediate. The function of both phosphatases in higher plants has been linked to inactivation of MAP kinases. AtPTP1, *Arabidopsis thaliana* Protein tyrosine phosphatase 1 has been shown to dephosphrylates tyrosine residue of activated AtMPK4 resulting in the loss of kinase activity (Huang *et al*., 2000). *AtPTP1* knockout mutant caused the enhanced activation of MAP kinases, whereas overexpression of *AtPTP1* resulted in delayed activation of these kinases (Luan, 2003). In addition, MAP kinase phosphatases (MKPs), a family of dual-specificity protein tyrosine phosphatases, specifically dephosphorylate both Thr and Tyr residues in the activation loop of MAP kinases.

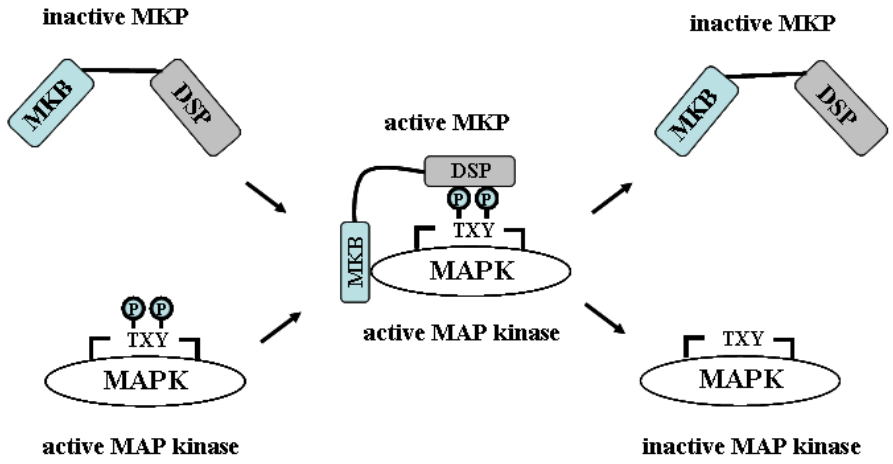


Figure 2. Interaction of MKPs with MAP kinase. According to Kondoh and Nishida (2007), with modifications. MKB, MAP kinase-binding domain; DSP, dual-specificity phosphatase domain.

MKPs containe the MAP kinase-binding (MKB) domain and dual-specificity phosphatases (DSP) domain. DSP domain plays a role of inactivation MAP kinase whereas MKB domain of MKPs regulates their enzymatic specificity via docking interaction with MAP kinases (Figure 2). *Arabidopsis* DsPTP1 and MKP1, alfalfa MP2C, and tobacco MKP1 have been shown to be responsible for the dephospholation of activated MAP kinase (Gupta *et al*., 1998; Ulm *et al*., 2001; Yamakawa *et al*., 2004).

1. 2 The complex signaling network through MAP kinases in plants

In the *Arabidopsis thaliana* genome, 20 genes for MAP kinases, 10 MAPK kinases, and more than 60 MAPKK kinases were identified (MAPK group 2002; Hamel *et al*., 2006). To date, functional data are available only for relatively a few of the corresponding proteins and their participation in different signaling pathway is largely unknown. Various members of the MAP kinase signal transduction pathways have been identified in plants in recent years. For example, cold, drought, and mechanical

stress activated AtMPK4 and AtMPK6 in *Arabidopsis* (Ichimura *et al*., 2000; Asai *et al*., 2002), and MsMMK in alfalfa (*Medicago sativa*) (Jonak *et al*., 1996; Bogre *et al*., 1997). The tobacco proteins SIPK and WIPK are the most extensively characterized plant MAP kinases. SIPK was identified as a protein kinase induced by salicylic acid in tobacco. Activation of SIPK has been shown by various abiotic and biotic stresses such as wounding, fungal elicitors, salicylic acid and infection with avirulent pathogen (Zhang and Klessig, 1997, 1998a, b; Zhang *et al*., 1998c; Romeis *et al*., 1999). Downstream targets of activated MAP kinases are largely unknown. In higher plants so far only 1-aminocylcopropane-1-carboxylic acid synthase (ACS), MKS1, WRKY and very recently EIN3 transcription factors are the only examples shown being phosphorylated by MAP kinases in *Arabidopsis* (Liu and Zhang, 2004; Andreasson *et al*., 2005; Menke *et al*., 2005; Yoo *et al*., 2008).

Several MAP kinases were identified recently from tomato. The tomato protein LeMPK2, highly homologous to SIPK from tobacco, was shown to be activated by an oligosaccharide elicitor and ultraviolet-B radiation. LeMPK3, highly homologous to WIPK from tobacco, was also shown to be activated by ultraviolet-B radiation, wounding, and infection by avirulant pathogens (Mayrose *et al*., 2004) and to be transcriptionally induced similar to WIPK (Kerry and Gregory, 2004). Computational analysis has revealed the presence of at least 16 members of MAP kinase families in tomato (Kerry and Gregory, 2004). Out of these sixteen members only those belonging to group A, namely LeMPK1, LeMPK2 and LeMPK3, were shown to be involved in hypersensitive responses (Stulemeijer *et al*., 2007) and systemin mediated the defense response against herbivorous insects (Kandoth *et al*., 2007).

Since the discovery of plant phytohormones, a number of evidences suggest the critical role in plant development and physiological responses (Hooykaas *et al*., 1999). Although the biosynthesis pathway of phytohormones is well known, our

understanding of the molecular mechanisms underlying phytohormones perception and signaling is limited. Resent studies have shown that MAP kinase cascades modulate and are modulated by phytohormones, and thus, suggest important roles of MAP kinase cascades in phytohormones signaling.

Abscisic acid (ABA) mainly acts to inhibit growth, promote dormancy, regulate the stomatal movement and help plant tolerate abiotic stress. ABA has been shown to induce transcription, translation, and activation of OsMAPK5 in rice (Xiong and Yang, 2003). In *Arabidopsis*, 42 kDa MAP kinase and AtMPK3 are activated by ABA. In ABA-hypersensitive *hyl1* mutant, both MAP kinases are activated at lower concentration of ABA than wild-type seedlings (Lu *et al*., 2002). ABA-regulated stomatal activity was inhibited by MAPK kinase inhibitor PD98059 in the epidermal peels from pea (Burnett *et al*., 2000). In addition, the sensitivity of *Arabidopsis* seeds to ABA was decreased in the presence of PD98059 (Lu *et al*., 2002). These results indicate the involvement of MAP kinase cascades in ABA signal transduction pathways and suggest that MAP kinase cascades may be the predominant transmission route for the ABA signal.

Ethylene is involved in the development processes and stress resistance. It has been shown to activate protein phosphorylation in tobacco and pea (Mishra *et al*., 2006). In *Arabidopsis*, ethylene activates a 47 kDa MAP kinase (Novikova *et al*., 2000). Overexpression of SIMKK (Alfalfa MAPK kinase) results in the constitutive activation of AtMPK6 and expression of ethylene-induced target gene (Ouaked *et al*., 2003). The activation of AtMPK6 leads to phosphorylate ACS2/6, and results in elevated level of ACS activity and ethylene production in *Arabidopsis* under stress condition (Liu and Zhang, 2004). NtMEK2-SIPK/WIPK cascades also regulate the induction of ethylene biosynthesis in response to wounding and viral infection (Kim *et al*., 2003).

SA, JA, and Oxylipins are important components of defense response pathways. JA plays a key role in the environmental stress responses and developmental processes of plants. SA is a signal molecule in the disease resistance-response of plants against pathogen attack. In tobacco suspension cells, SA was shown to induce a rapid and transient activation of SIPK (Zhang and Klessig, 1997). Oxylipins, another important signaling molecules in plants are formed both by enzymatic and non-enzymatic pathways. Important oxylipins formed by enzymatic reactions are 12-oxo-phytodienoic acid (OPDA) and jasmonic acid (JA), while those formed by non-enzymatic pathways includes hydrooxy fatty acids and phytoprostanes (Imbusch and Mueller, 2000; Mueller *et al*., 2008). Involvement of MAP kinase activation during oxylipin mediated signaling has been reported in photoautotrophic cell cultures of tomato (Thoma *et al*., 2003). Interestingly, activation in MAP kinase activity was observed due to phytoprostane elicitation while JA application did not result in the activation of MAP kinase. Though JA was shown to activate MKK3 (*Arabidopsis* MAPK kinase 3)-AtMPK6 cascades that negatively regulated ATMYC2 in JA-dependent gene expression and inhibition of root growth (Takahashi *et al*., 2007). In addition, JA has been shown to induce the activation of AtMPK1/AtMPK2 which belongs to subgroup C1 of Arabidopsis MAP kinases (Ortiz-Masia *et al*., 2007), but there was no activation of either SIPK or WIPK (homologues of MPK6 and MPK3, respectively) in tobacco (Kumar and Klessig, 2000). The transgenic tobacco plants co-suppressed the expression of *WIPK* gene fail to accumulate JA after wounding, whereas *WIPK*-overexpression plants exhibit elevated JA level (Seo *et al*., 1995, 1999). In addition, wound-induced JA production was reduced compared with wild-type plants in *WIPK*-, *SIPK*-, and *WIPK/SIPK*-silenced plants. Furthermore, wounding results in an abnormal accumulation of SA and transcripts for SA-responsive genes in *WIPK/SIPK*-silenced plants (Seo *et al*., 2007). These findings suggest that MAP

kinases are an important part of the JA and SA biosynthesis pathway.

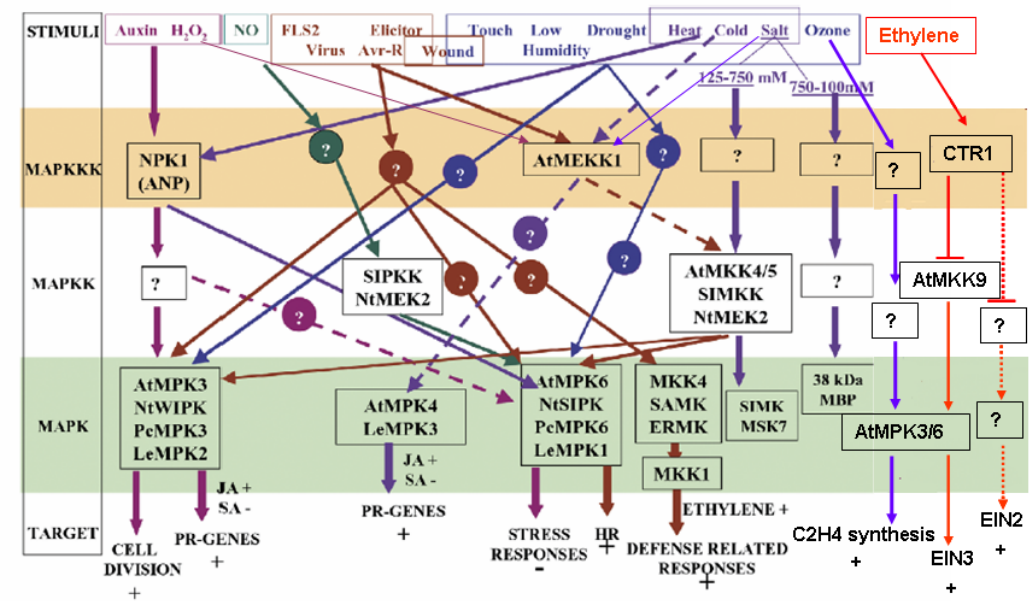


Figure 3 Schematic representation of cross-talk between different plant MAP kinase signaling pathway. According to Mishra *et al*., (2006), with additions. The homologs in Arabidopsis (At), Tobacco (Nt), Parsely (Pc), and Tomato (Le) are shown. ? indicated unidentified MAP kinase components.

Taken together, MAP kinases are regulated post-translationally by phosphorylation and dephosphorylation, and their function depends on the kinetics and amplitude of their activation. MAP kinase cascades are the complex networks rather than linear pathways (Figure 3). These networks are necessary for many fundamental physiological functions like stress signaling, hormonal response, and defense mechanisms.

1. 3 Protein kinase inhibitors are useful tools to probe the physiological function of MAP kinases

Protein kinases are one of the major mechanisms for controlling intracellular response to extracellular information and ultimately regulate the cellular responses such as proliferation, differentiation, secretion, apoptosis and also cell growth and development. Members of the protein kinase family can be categorized into three distinct classes based on the reversible transfer of the gamma-phosphate from ATP to specific serine, threonine, or tyrosine residues; the serine/threonine protein kinase, the tyrosine protein kinase, and the dual specificity protein kinase.

Analysis of the downstream mechanisms mediated by protein phosphorylation events including MAPK cascades in animals has been aided by the development of synthetic small molecules which have inhibition potential for a particular protein kinase (Cohen, 1999). The development of selective protein kinase inhibitors is a great importance for probing biological processes and promising approach for drug development. The ATP-binding site which is presented on a large hydrophobic surface has been focused for designing inhibitors that have exploited the differences in kinase structure and flexibility in order to achieve selectivity (Noble *et al*., 2004).

A number of compounds have been reported to inhibit particular serine/threonine protein kinases, and are being used extensively in cell-based assays to study physiological roles of the protein kinases that they are presumed to inhibit (Cohen *et al*., 1997; Monaco III *et al*., 2004). For example, the analogues of the anti-inflammatory agent pyridinylimidazole (SB [SmithLline Beecham] compounds) showed the highly potent and selective inhibitor effect on p38, but not ERK2, or other serine/threonine kinases (Wang *et al*., 1998). SB203580 which is one of SB compounds has been proposed to act in cells by stabilizing on inactive conformation

and result in reduction of p38 activation by MAPKKs (Knight and Shokat, 2005).

The purine analogues, roscovitine, olomoucine and bohemine, are used as potent inhibitors of cycline-dependent kinase (CDK) (de Azevedo *et al*., 1997), and were also shown to inhibit an alfalfa MAP kinase (Binarova *et al*., 1998) and tomato MAP kinases (Link *et al*., 2002a).

1. 4 MAPK signaling cascades regulate development and movement of stomata

Photosynthetic reactions may conveniently be divided into two phases, the light-dependent reactions and the light-independent reactions (carbon-fixing reactions). Stomata in the leaf epidermis provide the major pathway for gas exchange such as absorption of CO_2 from atmosphere and release of O_2 to their environment. Stomata formed by two guard cells surrounding a pore, open in response to a low concentration of CO_2 and close in response to elevated levels of CO_2. The turgor pressure in the guard cells is mediated by ion and organic solute concentration (Assmann, 1999). High level of CO_2 stimulate the activity of outward and inward rectifying K^+ channels and enhance S type anion channel activities (Ainsworth and Rogers, 2007). These changes cause stomatal close. Recent studies have revealed that some of protein phosphorylation events are related with stomatal movement and development (Hashimoto *et al*., 2006; Wang *et al*., 2007).

Arabidopsis HT1, which has high homology to MAPKK kinase, mutants are altered in their ability to control the stomatal movement in response to CO_2 (Hashimoto *et al*., 2006). YODA (YDA; MAPKK kinase) disrupt stomatal patterning and result in clustered stomata (Shpak *et al*., 2004). In addition, MKK4/MKK5-MPK3/MPK6 cascade loss-of-function mutants have shown the clustered stomata (Wang *et al*.,

2007). More recently, NtMPK4, tobacco homolog of Arabidopsis MPK4, was identified and shown to regulate the development of stomata and the activation of anion channels in guard cells (Gomi et al., 2005; Marten et al 2008). Taken together, these finding suggest that complete MAPK signaling cascades are key regulators of stomata development and movement.

1. 5 MAP kinases and primary metabolism

Sucrose, the major form of translocated carbon in higher plants, is transported by a concentration gradient from source to sink tissues. The carbon of sucrose is used for internal regulation and physiological responses that govern growth, development, sink metabolism and defense response (Koch, 1996; Roitsch, 1999; Berger *et al*., 2007).

Utilization of sucrose requires either irreversible hydrolyses by invertase (EC 3.2.1.26, β-fructofuranosidase) or reversible cleavage by sucrose synthase. There are several lines of evidences suggesting that the extracellular, cell wall-bound invertase isoenzymes are transcriptionally induced by a number of stimuli (Roitsch *et al*., 2003; Roitsch and Gonzalez, 2004). The stimuli that regulate extracellular invertase are also known to affect the carbohydrate requirements such as phytohormones, sugars, pathogen infection, and other stress-related stimuli (Figure 4) (Roitsch *et al*., 2003; Roitsch and Gonzalez, 2004).

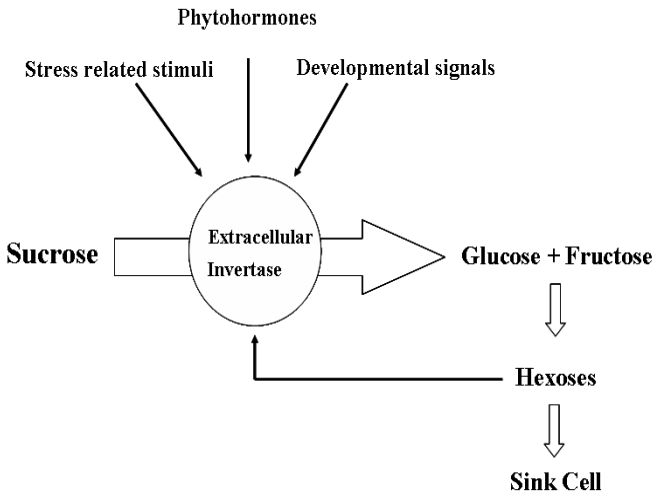


Figure 4. The regulation of exracellular invertase by sucrose. Extracellular invertase is important for supplying carbohydrates to sink tissue, and play a crucial role to mediate source/sink regulation in response to a variety of stimuli. According to Roitsch (1999), with modifications.

In autotrophic suspension cells of *Chenopodium rubrum*, and *Lycopersicon peruvianum,* the mRNA of extracellular invertase was induced by different stress-related stimuli, such as the fungal elicitor, protein phosphatase inhibitor, metabolizable and non-metabolizable sugars and mechanical wounding (Ehness *et al*., 1997; Sinha *et al*., 2002). Several isoforms of extracellular invertase have been identified from tomato (Godt and Roistch, 1997), carrot (Sturm and Chrispeels, 1990), Arabidopsis (Tymowska-Lalanne and Kreis, 1998), maize (Ying *et al*., 1999), and tobacco (Goetz *et al*., 2001). In tomato, extracellular invertases were shown to be encoded by a family of four different genes, *Lin5*, *Lin6*, *Lin7* and *Lin8* that are tissue specifically expressed and differentially regulated (Roitsch *et al*., 2003, 1999; Sinha *et al*., 2002; Proels *et al*., 2003, 2006). These results suggest that extracellular invertase is important for inducing heterotrophic metabolism in response to stress related stimuli.

It has been shown that extracellular invertases are part of a signaling network that results in co-ordinated regulation of source-sink relations and defense responses

(Ehness *et al*., 1997; Sinha, PP). The finding that different stress related stimuli result in the differential activation of available MAP kinases indicate that MAP kinases may be involved in the integration of stress related stimuli to co-ordinate direct defense responses with the regulation of primary carbon metabolism.

1. 6 Tocopherol interfere with the activation of MAP kinases

Reactive oxygen species (ROS) derived from molecular oxygen can accumulate in plant under a variety of biotic and abiotic stress conditions and cause oxidative damage to the cellular compounds including proteins, chlorophyll and lipids. To survive in these conditions plants have developed two general protective mechanisms; enzymatic and non-enzymatic detoxification (Alscher and Heath, 2002). Tocopherols, together with tocotrienols better known as vitamin E, are lipophilic anitioxidants synthesized by plants, algae, fungi and some cyanobacteria. There are four natural analogues of tocopherols, α, β, γ, and δ, which have different bioactivities. α-tocopherol commonly accumulates in the leave of plants whereas γ-tocopherol is rich in seeds. β-and δ-tocopherol are not very abundant in most plant species. In addition, α-tocopherol has been proposed to participate in the detoxification of ROS together with the hydrophilic antioxidants glutathione and ascorbate (Foryer and Noctor, 2003). The physiological function including antioxidant agent of tocopherol has been shown in plants. The tocopherol deficient mutant *vte2* showed defective in transfer cell wall development and photoassimilate transport at low temperature (Maeda *et al*., 2006), reduced seed viability and impaired seeding development (Sattler *et al*., 2004). In addition, the overexpression of tocopherol cyclase enhanced tolerance to drought stress and resulted in accumulation of tocopherol (Liu *et al*., 2008). Furthermore, the maize *Suc export defective 1* (*sxd1*) mutant and StSXD1

(*Solanum tuberosum sxd1*) knockdown potato resulted in tocopherol deficiency and the accumulation of carbohydrates (Russin *et al*., 1996; Hofius *et al*., 2004). A similar carbohydrate phenotype (i.e., the accumulation of starch, sucrose, glucose and fructose) occurred in the orthologous Arabidopsis *vte1* and *vte2* mutant during low-temperature treatment, whereas both mutant plants did not show similar carbohydrate phenotype under favorable condition (Maeda *et al*., 2006). These finding suggest the link between tocopherol pathway and primary carbohydrate metabolism, although the mechanism is unclear.

Resent studies suggested that tocopherol modulates the activation of specific enzymes involved in signal transduction pathways in mammalian cells. The tumour necrosis factor (TNF)-α-induced activation of MAP kinases like ERK 1/2 and p38 was inhibited by α-tocopherol in bronchial epithelial cell lines (Ekstrand-Hammarstroem *et al*., 2006). The similar effect of α-tocopherol on phorbol myristate acetate (PMA) induced-ERK 1/2 activation in smooth muscle cells has been reported by Clement *et al*., (2002). In addition, α-tocopherol induced the activity of phosphoserine-threonine phosphatase (PP2A) and phosphotyrosine phosphatases (PTP) (Zingg, 2007). Taken together, these findings suggest that the effect of α-tocopherol on the inactivation of stimuli-induced MAP kinases may be mediated by the increased activity of phosphatases such as PP2A and PTP and indicate that tocopherol, particularly α-tocopherol, may regulate a number of non-antioxidant functions. However, most studies of tocopherol actions have been focused on antioxidant agent, while the non-antioxidant functions are still poorly understood in plants.

1. 7 The role of PLAT domain proteins in plant defense response

Plants activate multiple defense mechanism in response to various biotic (i.e., pathogen infection and insect herbiory) and abiotic (i.e., high and low temperature, drought, ozone, UV light, salinity, and osmotic stress) stresses to protect them selves. To survive these conditions plants have developed a complex signaling mechanisms such as the synthesis of hormones like abscisic acid (ABA), ethylene, jasmonic acid (JA), and salicylic acid (SA), programmed cell death, the rapid production of reactive oxygen species (ROS) and transcriptional activation of defense genes.

The ROS including hydrogen peroxyde (H_2O_2), superoxide anion, and hydroxyl radicals is commonly accumulated and/or generated by different abiotic and biotic stresses. H_2O_2 is an active signaling molecule and its accumulation and concentration lead to a variety of cellular responses. High level of H_2O_2 results in a hypersensitive cell death. On the other hand, it can act as a diffusible signal for the induction of protecting genes adjacent to HR lesions, thereby limiting oxidant-mediated cell death (Orozco-Cardenas et al., 2001).

The expression of many defense related genes is regulated by signaling molecules such as ABA, JA, ethylene, and SA. Antagonistic interaction between ABA and JA-ethylene signaling pathway has been reported by Anderson *et al*., (2004). This indicates the complexity of cross talking between various signaling pathways during stress adaptation. SA has been shown to be important components of defense response pathways (Reymond and Farmer, 1998), and its accumulation has been shown to be associated with the activation of defense genes in many plant species (Durner *et al*., 1997; Dempsey *et al*., 1999). SA activates the systemic, long-lasting, systemic acquired resistance (SAR) and regulates the expression of acidic PR protein such as PR1, PR2, and PR5, and also the apoptotic-like cell death, like HR.

In *Arabidopsis*, only half of the *ca*. 27000 genes have been functionally annotated based on sequence similarities to known genes. Among these, the experimental evidence concerning their function is available only for 11 %. Therefore, the functional elucidation of unknown genes is one of the major challenges in plant science. On the basis of the fully sequenced *Arabidopsis* genome, the PLAT (Polycystin-1, Lipoxygenase, and Alpha-toxin) domain is present in a variety of membrane or lipid associated proteins not only as multi-domain proteins in case of polycystin, lipoxygenases, alpha-toxin and lipases, but also as a single domain or as repeats. The importance of PLAT domains is demonstrated by mutations in lipoprotein lipase or tiacylglycerol lipase in human (Hegele *et al*., 1992). In addition, the yeast two-hybrid (Y2H) screen using cDNA library derived from mix-staged him-5 animals suggests that the PLAT domain of *C. elegans* polycystins LOV-1 and Human polycystin-1 interact with ATP-2, an ATP synthase F1 subunit (Hu and Barr, 2005). Furthermore, it has been suggested that PLAT domain may function to localize the enzyme near its membrane or lipoprotein sequestered substrates by lipase-procolipase or protein-protein interaction in rabbit 15-lipoxygenase, and directly interact with the membrane in a Ca^{2+} dependent manner in plant lipoxygenases (Bateman and Sandford, 1998). However, the molecular function and biological process of single PLAT domain proteins are largely unknown in higher plants.

In former study, AtPDP1 (*Arabidopsis thaliana* PLAT domain protein 1) was purified from intracellular fraction of *Arabidopsis* mixotrophic cultured cells treated with 1mM SA. In addition, the fraction which contained AtPDP1 showed high level of antimicrobial activity against *C. maltosa* (Ali, 2007). AtPDP1 contains the single PLAT domain and belongs to the PLAT-plant-stress subgroup including elicitor inducible protein from tobacco (Accession number: BAB13708) and rice (Accession number: NP_921022), TMV-induced protein 1 (Accession number: AAF63515), and

dehydration stress-induced protein from rape (Accession number: AAK01359), rice (Accession number: BAD37679), and *Arabidopsis* (Accession number: AAM65891). Many of members in this subgroup are induced by stress. *CaTin1* (*Caspsicum annuum* TMV-induced clone 1) which contains a PLAT domain was expressed by ethylene but not by MeJA and SA (Shin *et al*., 2004). In addition, overexpression of *CaTin1* resulted in big HR lesion formation after TMV inoculation and has been shown enhanced tolerance against various stresses such as drought and salt stress. In higher plants so far only *CaTin1* is the only example showing the physiological function of PLAT domain proteins.

1.8 Scope of work

Abiotic and biotic stress-related stimuli activate different signal transduction pathways that are ultimately integrated to coordinately regulate gene expression. Activation of different subsets of MAP kinases provides a mechanism to integrate different signals, and results in coordinated and signal-specific gene regulation. Plant defense mechanisms via MAP kinase signaling have been addressed by a number of studies, whereas interaction of MAP kinase signaling with primary metabolism is poorly understood.

Sugars have important functions as primary messengers in signal transduction and essential roles as substrates in carbon and energy metabolism. Previously, it was shown that the expression of extracellular invertase can be blocked by the kinase inhibitor straurosporine, whereas the phosphatase inhibitor endotall induces the expression of extracellular invertase (Ehness *et al*., 1997). Based on this finding, we hypothesized that MAP kinase signaling may be a possible upstream signal of primary metabolism in plants. However, further experiments are required to

determine the role of MAP kinase signal in primary metabolism. Therefore, we investigate the possible relationship between the transcriptional regulation and posttranslational activation of MAP kinases and the stress-related stimuli dependent induction of primary metabolism. In a first step, the regulation of MAP kinases with induction of extracellular inverase (Lin 6) has been investigated by a functional approach. To assess the role of Lin6 in stress signal transduction by MAP kinases, transgenic tobacco plants transformed with the GUS reporter gene under the control of Lin6 promoter were transiently transformed by a His-tagged version of LpMPK2 and a HA-tagged version of LpMPK3 under the control of CaMV35S promoter. Looking at the signaling properties of sugars, the photoautotrophic cell culture of *Arabidopsis thaliana* was used effectively to study the fast signal transduction process by studying the activation and identification of specific MAP kinases by glucose signaling. The application of a biochemical purification technique allows the identification of the associated MAP kinases. To perform a causal analysis of MAP kinase action in the photosynthesis, we identified the CO_2-induced MAP kinases activation. The finding of CO_2-induced MAP kinases in tobacco allowed us to demonstrate that elevated levels of CO_2 activated NtMPK4 and SIPK, and resulted in the stomata movement. As mentioned above, the non-antioxidant function of α-tocopherol in the stress induced signaling pathways has been suggested in mammalian cells. Surprisingly, it has been shown that *Arabidopsis thaliana* vitamin E biosynthetic mutant (tocopherol deficient mutant) *vte1* and *vte2* exhibited the dramatic carbohydrate phenotypes under low temperature (Maeda et al., 2006). This finding prompted us to investigate the possible link between the physiological function of α-tocopherol and primary carbon metabolism under stress induced MAP kinase signaling.

In plant, the gain-of-function and the loss-of-function mutants are commonly used for

the study of the physiological roles of MAP kinases. There is no report so far on developing of inhibitors, which would be useful tools for probing biological processes and understanding physiological roles of MAP kinase signaling. Thereby, the focus was directed towards the development of MAP kinase inhibitor using the purine analogues in this thesis. This basic characterization of the purine analogues suggests that the selectivity and potency of inhibitors can be improved by the modification of chemical structure.

Many of single PLAT domain proteins which belong to the PLAT-plant-stress subgroup members are induced by stress. However, very little is known about the physiological role of single PLAT domain proteins in higher plants. In this study, *Arabidopsis thaliana* PLAT domain protein 1 (AtPDP1) was used in a functional approach to elucidate the physiological role of AtPDP1 in stress response pathways.

The present thesis provides basic information about interaction of sucrose metabolism with stress signal transduction pathway and provides the basis for future research on the regulation of different defense signaling pathways.

2. Materials and methods

2. 1 Materials

2. 1. 1 Chemicals

Chemicals used in experiment were bought from Amersham (Buckinghamshire, UK), Diagonal (Muenster), Macherey-Nagel (Dueren), Aldrich (Steinsheim), Fluka (Neu-Ulm), Merck (Darmstadt), Roth (Karlsruhe), AppliChem (Darmstadt) or Sigma (Taufkirchen).

DNA-Polymerase (Taq) was from Segenetic (Borken) and other enzymes like restriction enzyme were from Fermentas (St. Leon-Roth).

2. 1. 2 Medium

LB Medium	10 g L^{-1} Trypton, 5 g L^{-1} Yeast extract, 10 g L^{-1} NaCl : pH 7.0
LB Medium Plate	LB medium with 15 g L^{-1} Bacto-agar
YEB Medium	5 g L^{-1} Beef extract, 5 g L^{-1} Pepton, 1 g L^{-1} Yeast extract, 5 g L^{-1} Sucrose, 0,5 g L^{-1} MgSO4 : pH 7,2
PDA Medium	39 g L^{-1} Potato Dextrose Agar
MS Medium	4.4 g L^{-1} MS, 20 g L^{-1} Sucrose : pH 5.7 3 g L^{-1} Phytogel
King´s Medium B	2 % Peptone, 1 % Glycerol, 8 mM K_2HPO_4, 6 mM $MgSO_4$

2. 1. 3 Buffers

2. 1. 3. 1 Protein Extraction Buffer

Extraction buffer (for tobacco and tomato)	100 mM HEPES pH 7.5, 5 mM EGTA, 5 mM EDTA, 10 mM DTT, 10 mM Na_3VO_4, 50 mM β-Glycerophosphate, 10 % (w/v) Glycerol 0.1 mM PMSF, 1 mL L^{-1} Protease inhibitor Cocktail, 1 mM BA
Extraction buffer (for *Arabidopsis*)	25 mM Tris pH 7.5, 5 mM $MgCl_2$, 5 mM EDTA, 1 mM DTT, 5 % (w/v) Glycerol, 10 mM NaF 20 mM β-Glycerophosphate, 1 mM Na_3VO_4 1 mM PMSF, 10 mM BA 1mL L^{-1} Protease inhibitor Cocktail

2. 1. 3. 2 Bradford method

BSA standard	100 µg mL^{-1} BSA in H_2O
1x Brad ford bye (100 mL)	10 mg Coomassie Brilliant blue G-50 5 mL EtOH (95%), 10 mL Phosphoric acid (85%)

2. 1. 3. 3 SDS PAGE

SDS-Loading buffer	25 mM Tris, 192 mM Glycin, 0.1 % SDS	
10x sample buffer	10 % SDS, 45 % (w/v) Glycerol, 0.5 M Tris pH 6.8 0.2 % Bromphenol blue, 0.2 M DTT	
15% Separating Gel (for 5mL)	H_2O	1.1 mL
	30% acrylamide mix	2.5 mL
	1.5 M Tris pH 8. 8	1.3 mL
	10% SDS	0.05 mL
	10% ammonium persulfate	0.05 mL
	TEMED	0.002 mL

5 % Stacking Gel	H_2O	1.4 mL
(for 2 mL)	30% acrylamide mix	0.33 mL
	1.0 M Tris pH 6. 8	0.25 mL
	10% SDS	0.02 mL
	10% ammonium persulfate	0.02 mL
	TEMED	0.002 mL

2. 1. 3. 4 Chromatographic Buffer

Buffer A	25 mM Tris pH 7.5, 5 mM $MgCl_2$, 5 mM EDTA, 1 mM DTT, 5 % (w/v) Glycerol, 10 mM NaF 20 mM β-Glycerophosphate
Buffer B	Buffer A with 1 M NaCl

2. 1. 3. 5 In Solution assay

Reaction buffer (for in solution assay)	25 mM Tris pH 7.5, 5 mM $MgCl_2$, 25 µM ATP 0.5 mg mL-1 MBP, 1 mM EGTA, 1 mM DTT, 1 µCi [γ^{32}P]ATP
Reaction buffer (for inhibitor assay)	75 mM Tris pH 7.5, 15 mM $MgCl_2$, 75 µM ATP, 1.5 mg mL-1 MBP, 3 mM EGTA, 3 mM DTT, 3 µCi [γ^{32}P]ATP

2. 1. 3. 6 In Gel assay

Separating Gel	H_2O	660 µL
(for 2mL)	30% acrylamide mix	0.7 mL
	1.5 M Tris pH 8. 8 with 10 % SDS	0.525 mL
	10% ammonium persulfate	25 µL
	TEMED	1 µL
	5 mg mL^{-1} MBP	125 µL
Stacking Gel	H_2O	3.7 mL
(for 4 mL)	30% acrylamide mix	0.8 mL

1.0 M Tris pH 6. 8 with 10% SDS	1.5 mL
10% ammonium persulfate	50 µL
TEMED	15 µL

Renaturation Buffer 25 mM Tris pH 7.5, 0.5 mM DTT, 0.1 mM Na_3VO_4, 5 mM NaF

Wash buffer Renaturation Buffer with 0.5 mg mL^{-1} BSA and 0.1 % Triton X-100

Reaction buffer 25 mM Tris pH 7.5, 12 mM $MgCl_2$, 200 nM ATP
2 mM EGTA, 1 mM DTT, 0.1 mM Na_3VO_4
50 µCi [γ^{32}P]ATP

Wash buffer 5 % Trichloroacetic acid, 1 % NaPPi

2. 1. 3. 7 Immuno complex kinase activity assay

Reaction buffer with antibody
57 µL crude extract
1.2 µL Nonident P-40 (5%)
0.9 µL NaCl (5 M)

Wash buffer (100 mM NaCl)
20 mM Tris pH 7.5, 5 mM EDTA, 100 mM NaCl
1 % Triton X-100

Wash buffer (1 M NaCl) 10 mL wash buffer + 1.8 mL 5M NaCl

Reaction buffer (for wash)
20 mM HEPES pH 7.5, 15 mM $MgCl_2$, 5 mM EGTA
1 mM DTT

Reaction buffer 20 mM HEPES pH 7.5, 15 mM $MgCl_2$, 25 µM ATP
5 mM EGTA, 1 mM DTT, 0.5 mg mL^{-1} MBP
1 µCi [γ^{32}P]ATP

2. 1. 3. 8 GUS fluorimetric assays

0.1 M $NaPO_4$ (500 mL) 28.9 mL Na_2HPO_4 (1M), 21.2 mL NaH_2PO_4 (1 M)

Extraction buffer	50 mM $NaPO_4$, pH 7.0, 10 mM EDTA 0.1 % Triton X-100, 0.1 % N-lauroysarcosine 10 mM β-mercaptoethanol
MUG	2 mM MUG in extraction buffer
Stop buffer	1 M Sodiumcarbonat
MU standard	1 mM MU in stop buffer

2. 1. 3. 9 Plant transient expression assays

AB medium	3.9 g L^{-1} MES, 10 g L^{-1} glucose : pH 5.5
20X AB Buffer	60 g L^{-1} K_2HPO_4, 20 g L^{-1} NaH_2PO_4
20X AB Salts	20 g L^{-1} NH_4Cl, 6 g L^{-1} $MgSO_4$-$7H_2O$, 3 g L^{-1} KCl 3 g L^{-1} $CaCl_2$, 3 g L^{-1} $FeSO_4$-$7H_2O$
Induction medium	940 mL AB medium, 50 mL 20X AB salts 10 mL 20X AB buffer, 100 µM Acetosyringone

2. 1. 3. 10 RNA extraction and Blotting

RNA-denaturing solution	4 M Guanidinium-thiocyanat 25 mM (tri) Na-citrate 0.5 % Sarcosyl (N-laury-sarcosine) 7 µL mL^{-1} β-mercaptoethanol
Na-acetate solution	2 M Na-acetate pH 4.0
CIA (250 mL)	240 mL chloroform, 10 mL isoamyl alcohol
RNA-loading buffer	76 µL 5x RNA-Running Buffer, 126 µL Formaldehyd 378 µL Formamid, 76 µL 10X DNA-Loading buffer 5 µL Ethidiumbromid (5mg mL^{-1})

RNA-Running Buffer (5X)	200 mM MOPS, 50 mM NaAc, 5 mM EDTA, 0,1 % DEPC : pH 7.0
1.2 % formaldehyde agarose gel	1.2 g agarose in 62.2 mL H_2O : melt in microwave oven 20 mL 5X RNA-Running Buffer 17.8 mL 37 % Formaldehyd
20X SSC buffer	3 M NaCl, 0.5 M Na-Citrate : pH 7.0
50X Denhardts Solution	1 % Polyvinypyrrolidone K 30(PVP K30) 1 % BSA, 1 % Ficoll 400
20X SSC	3 M NaCl, 300 mM Na-Citrat : pH 7.0
Hybridization buffer	50 % Formamide, 5X SSC, 5X Denhardts solution 0.1 % SDS, 5 mM EDTA, 100 µg Salamen sperm DNA
Wash buffer I	2X SSC, 0.1 % SDS
Wash buffer II	0.2X SSC, 0.1% SDS

2. 1. 4. Computer software and Internet address

Microsoft® Word 2003, Excel 2003, and PowerPoint 2003

Adobe Photoshop 7.0

AIDA software

Vector NTI Suite 6.0

Spot Advanced Version 3.5.5

ImagingWin

http://www.ncbi.nlm.nih.gov

http://www.arabidopsis.org

http://www.genevestigator.ethz.ch

http://www.cbs.dtu.dk/services/TargetP/

2. 2 Methods

2. 2. 1 Plant Cell cultures

Tomato: Photoautotrophic suspension culture cells of *Lycopersicon peruvianum,* described previously by Beimen *et al*. (1992) were used experimental system. For the stress treatment, suspension cell cultures were sub-cultured for 2 weeks in Murashige and Skoog medium (Duchefa, http://www.duchefa.com), and incubated shaking under continuous light conditions with an atmosphere containing 2% (w/v) CO_2.

Arabidopsis: Photoautotrophic suspension culture cells were subcultured every 2 weeks in B5 medium containing 1mg L^{-1} of 2,4-D and incubated shaking under continuous light conditions with an atmosphere containing 2 % (w/v) CO_2. Cells were used for the experiments during the second week after sub-culturing. To ensure same starting conditions for all cultures of an experiment, cultures were mixed and divided again 24 h before treatment.

***Arabidopsis* (mixotrophic suspension culture cells)**: Suspension cell cultures were sub-cultured for 2 weeks in B5 medium (Duchefa, http://www.duchefa.com), and incubated shaking under continuous light conditions.

2. 2. 2 Plant growths

Stable transgenic tobacco plants (*Nicotiana tabacum* cv. Samsun NN) expressing *AtPDP1* gene, tobacco plants (*Nicotiana tabacum* L. cv. SR1) expressing GUS reporter gene under the control of *Lin6* promoter, and wild-type *Nicotiana benthamiana* and *Nicotiana tabacum* var. Samsun were grown at 25^{o}C in the green

house. All experiments were performed using 2nd true leaves of 4 week old plants.Wild-type Arabidopsis thaliana Col gl1 were growth in soil at 9 h light period (light intensity: 180 umol quanta $m^{-2}s^{-1}$).

2. 2. 3 Stress treatments

Oxylipins: OPDA which was synthesized from linolenic acid using linseed acetone powder and purified by HPLC, MeJA, and Hexenal were a generous gift from M. J. Mueller (University of Wuerzburg). The phytoprostanes A_1 (PPA_1) was prepared as described previously (Thoma et al., 2003). Prostaglandin A_1 (PGA_1) was obtained from Biomol (Biomol, www.biomol.de). Hydroxy nonenal was obtained from Biozol. All oxylipins were evaporated under a gentle stream of nitrogen and immediately resolved in methanol, or diluted in methanol before administration.

CO_2 Treatment: *Nicotiana tabacum* var. Samsun plants were grown in the green-house. All experiments were performed using 2nd true leaves of 4 week old plants. 2 hours before treatment with CO_2, we took and kept the leaf in CO_2-free condition. Guard cells were stimulated by switching from CO_2-free air to air containing 700 µL L^{-1} CO_2 (Roelfsema et al., 2002).

Wound stress: the leaf of tobacco was lightly pressed for 3 times by a gloved hand. Other stimuli were applied at the following concentration unless indicated differently

Stress-related stimuli	Concentration
E-Fol	100 g mL^{-1}
PGA	100 g mL^{-1}
KCl	50 mM
SA	1 mM
MeJA	75 µM or 100 µM

D-glucose	50 mM
Mannitol	50 mM
OPDA	75 µM
PPA_1	75 µM
Hexenal	75 µM
Hydroxy nonenal	75 µM
PGA_1	100 µM
Cold	6 ^oC

The cells were harvested at different time points by centrifugation, snap freezing in liquid nitrogen and stored at -80^oC until analysis.

2. 2. 4 Pathogen infections

***Pseudomonas syringae*:** Bacteria were inoculated into 10 mL King's B medium with antibiotics and grown overnight at 28 ^oC. The pellet of bacteria was collected by centrifugation at 3000 rpm for 10 min, and washed three times with 10 mM $MgCl_2$. Different concentration of Bacteria were infiltrated into plant leaves using 1 mL needleless syringe.

Antibiotics concentration	
Pseudomonas syringae pv. *tomato* DC3000	50 mg L^{-1} Rifampicin
Pseudomonas syringae pv. *tomato DC3000 RPM1*	50 mg L^{-1} Rifampicin
Pseudomonas syringae pv. *tabaci (tetracycline resistance)*	20 mg L^{-1} Tetracycline
Pseudomonas syringae pv. *tabaci (GFP labeled)*	50 mg L^{-1} Kanamycin

S. sclerotiorum: S. sclerotiorum were growth on PDA medium at RT for 1 week under darkness condition. For inoculation, an agar plug (0.4 cm in diameter) containing the advancing edge of growing mycelia was placed in the center of leaf. Infected plants were kept in a clear plastic box under saturating humidity at 22 ^{o}C under white fluorescent light.

2. 2. 5 Plasmid DNA isolation

Bacteria were inoculated into 3 mL LB medium with antibiotics and grown overnight at 28 ^{o}C. The pellet of bacteria was collected by centrifugation at 12000 rpm for 2 min, resuspended completely in 100 µL of Solution 1, added 200 µL solution 2, and mixed by tapping tube with finger. After mix, 150 µL of solution 3 was added into the tube. Bacterial lysate was collected by centrifugation at 12000 rpm for 10 min at 4 ^{o}C, and supernatant was transferred to new tube. Plasmid DNA pellet precipitated by addition of EtOH, and collected by centrifugation at 12000 rpm for 10 min at 4 ^{o}C. The pellet was washed with 70 % EtOH, and dried at RT for 20 min.

Solution 1	50 mM glucose, 25 mM Tris pH 8.0, 10 mM EDTA pH 8.0	
Solution 2	0.2 N NaOH, 1 % SDS	
Solution 3	5 M potassium acetate	60 mL
(100 mL)	Glacial acetic acid	11.5 mL
	H_2O	28.5 mL

2. 2. 6 Plasmid Constructions

Total RNA isolated from cell cultures of *Lycopersicon peruvianum* was reverse-transcribed using a ReverAid^{TM} First strand cDNA synthesis kit (Fermentas, http://www.fermentas.com). A 1184 bp DNA fragment of LpMPK2 was amplified from

cDNA by using primer Xho I-LpMPK2F and Xba I-LpMPK2R) which are designed based on the sequence in the NCBI data-bank (accession number : AY261513, http://www.ncbi.nhm.nih.gov.). The PCR-amplified product was digested with Xho I and Xba I and cloned into Xho I/ Xba I-digested pPS1 binary vector.

DNA-digestion condition

10x Reaction Buffer	2.5 µL	: Incubation at 37°C for 2-3 hours
Plasmid DNA	0.5-15 µg	
Restriction enzyme (10 U μL^{-1})	0,5 – 2 µL	
H2O	to 25 µL	

Ligation condition

10x T4 ligase buffer	1 µL	: Incubation at 16°C for ON
Digested Vector	50 ng	
Insert	150 ng	
T4 DNA ligase	1 µL	
H2O	to 10 µL	

For cloning LpMPk3, using the same cDNA as template, a cDNA containing the 5' untranslated region was amplified by PCR with LpMPK3-F and LpMPK3-Rev primers designed based on the sequence in the TIGR EST data-bank (TC85947 and TC872609, http://www.tigr.org). The PCR-amplified product was cloned into that site of pSLF172 to produce a C-terminal tripe HA-tagged LpMPK3. A 322bp region LpMPK3 (3’ untranslated region) was amplified by PCR with LpMPK3-RNAi-F and LpMPK3-RNAi-Rev primers, inserted into the pHannibal vector and mobilized into the corresponding binary vector pART27.

Total RNA isolated from cell cultures of Arabidopsis thaiana (Columbia ecotype) was reverse-transcribed using an ReverAidTM First strand cDNA synthesis kit (Fermentas, http://www.fermentas.com). Using this cDNA as template, a cDNA was amplified by PCR with AtPDP1-F and AtPDP1-Rev primers designed based on the sequence in

the NCBI data-bank (Accession number: At4g39730, http://www.ncbi.nhm.nih.gov). The PCR-amplified product was cloned into pPS1 binary vector or into pSAT1396-gfp vector.

PCR-Reaction Cocktail

DNA	100 ng
Primer-F (100 pmol μL^{-1})	1 µL
Primer-Rev (100 pmol μL^{-1})	1 µL
dNTP-mix (10 mM)	1 µL
MgCl2 (25 mM)	1-3 µL
Taq (0.5u μL^{-1})	1 µL
10xPCR Buffer	2.5 µL
H_2O	25 µL

PCR running programme

Process	Temperature (^oC)	Time	Number of cycle
Denaturation	94	5 min	1
Denaturation	94	1 min	
Annealing	55-58	1 min	40
Elongation	72	1 min -2 min	
Extension	72	10 min	1

Primer list

Name	Primer sequence
Xho I-LpMPK2F	5’-CTCGAGATGGATGGTTCACCGCAAACG-3’
Xba I – LpMPK2R	5’-TCTAGATTAATGATGATGATGATGATGCATGTGCTGGTATTCGGGATTAAATGCAAGACCCTC-3’ (6x His tag sequence is underlined in the reverse primers)
LpMPK3-F	5’-CTCGAGCTCTTCTTCTTCTCATCTTCC-3’
LpMPK3-Rev	5’-GCGGCCGCCAGCATATTCAGGATTCAAC-3’
LpMPK3-RNAi-F	5’-GAATTCGGATCCATGCTTAAGCATAAGAGAAATCAG-3’
LpMPK3-RNAi-Rev	5'-CTCGAGTCTAGATACA-AGTCATATGAATGAGTG-3'
AtPDP1-F	5'-ATGGCTCGTCGCGATGTTCT-3'
AtPDP1-Rev	5'-TTAAACGACCCAAGAAAGC-3'

2. 2. 7 Plant transient expression assays

A single colony of *A. tumefaciens* LBA4404 containing either His-tagged *LpMPK2* or empty vector, HA-tagged *LpMPK3* or *LpMPK3*-RNAi, or *AtPDP1* or empty vector construct was inoculated into 10 mL induction medium with antibiotics and grown overnight at 28 °C. The bacteria were collected by centrifugation and resuspended in 10 mM MES and 10 mM $MgCl_2$ containing 150 µM acetosyringone to an OD_{600} of 1.0. Fourth or fifth leaves of plants were infiltrated with the bacterial suspension using a sterile 1 mL needleless syringe. The infiltrated area was immediately outlined with a marker pen.

2. 2. 8 RNA extractions

Tomato cells : Total RNA were extracted by grinding cells in 500 µL RNA-denaturing buffer, 50 µL NaAc-solution, and 500 µL acid-phenol. The homogenate was incubated at RT for 15 min and then 0.1 mL CIA was added. After centrifugation at 13000 rpm for 10 min, the supernatant was transferred into new tube, added 500 µL CIA, then incubated at RT for 10 min. After centrifugation, the upper phase was transferred to a new tube and the same volume of isopropanol was added. The sample was kept at -20 ^{o}C for overnight to allow RNA precipitation. RNA was collected by centrifugation at 13,000 rpm for 30 min at 4 ^{o}C. The RNA pellet was washed twice with 750 µL of 3 M $LiCl_3$ and once in 750 µL of 70 % ethanol. After centrifugation at 13000 rpm for 15 min at 4 °C, the RNA pellet was dissolved in 20 µl of DEPC-treated water.

***Arabidopsis*:** 1 mL TriFast was added to 100 mg grinding material and vortex at high speed for 15 sec. 5 min after incubation at RT, 200 µL of chloroform was added, and mix by vortex. The supernatant was separated by centrifugation at 11000 rpm for 10

min at 10 ^{o}C, and added 600 µL of isopropanol. The RNA pellet was collected by centrifugation at 13,000 rpm for 15 min at 4^{o}C. RNA pellet was washed twice with 750 µl of 3 M $LiCl_3$ and once in 750 µL of 70 % ethanol. After centrifugation at 13000 rpm for 15 min at 4°C, the RNA pellet was dissolved in 20 µl of DEPC-treated water.

2. 2. 9 RNA analyses

Total RNA (15 µg) was electrophoresed on a 1% formaldehyde agarose gel, transferred to a positively charged nylon membrane, and fixed by baking at 80°C for 2 h. The probe was prepared using DNA labeling kit (Fermentas, St. Leon-Roth). 150 ng of DNA was incubated with 10 µL of hexanucleotide buffer at 95°C. 10 min after incubation, sample was put on ice for 2 min, and then incubated with 3 µL Mix A, 5 µL α^{32}P-ATP, and 1 µL Klenow enzyme at 37 °C for 10min. After incubation, 4 µL dNTP mix was added into probe sample, and incubated at 37 °C for 10min. The labelled probe was purified by microspin column, and denatured by incubation at 95 °C for 5min. The membrane was incubated in hybridization buffer at 42 °C with shaking. 2 hours after incubation, the denatured probe was added to hybridization buffer, and incubated again for overnight at 42 °C with shaking. The non-binding probe was removed by washing with wash buffer I and II.

2. 2. 10 Protein extractions

Proteins were extracted by grinding leaf tissue or cells in extraction buffer. After centrifugation at 14000 rpm for 10 min, supernatants were transferred into new tubes, and stored at -80°C. The concentration of protein extracts was determined according to Bradford method (Bradford 1976) with bovine serum albumin as standard.

2. 2. 11 GUS fluorimetric assays

For GUS fluorimetric assays, total protein was extracted by grinding leaf tissue in GUS extraction buffer. After centrifugation at 13000 rpm for 10 min at 4 °C, supernatants were transferred into new tubes. 50 µL of protein extract was mixed with 50 µL of 2 mM MUG (methylumbelliferyl-β-D-glucuronide, Sigma, http://www.sigma-aldrich.com) in extraction buffer, and incubated at 37 °C. The reaction was stopped by the addition of 50 µL of Na_2CO_3. Fluorescence was measured at 355 nm excitation/ 460 nm emission using the Fluoroskan Ascent (Labsystem, http://www.labsystems.fi). GUS enzyme activity was expressed as picomoles of MU (4-methylumbelliferone, Sigma, http://www.sigma-aldrich.com) produced per minute per microgram of protein. All GUS measurements were confirmed in three independent experiments.

2. 2. 12 Immuno complex kinase activity assay

The immuno complex kinase assay was performed as described previously (Link *et al*., 2002a). Protein extract (100 µg) was incubated either with His (GE Healthcare, hptt://www.gehealthcare.com), HA11 (COVANCE, http://www.CRPinc.com), AtMPK3, AtMPK4, AtMPK6, or NtMPK4 antibody in immunoprecipitation buffer at 4°C for 2 hr. About 20 µL of protein A-agarose (50% suspension, Oncogene, http://www.oncogene.com) was added, and the incubation was continued for an additional 2 hr at 4 °C. The complexes were precipitated by a brief centrifugation and washed three times with wash buffer, one time with wash buffer containing 1 M NaCl, and one time with reaction buffer. Kinase reactions were performed for 30 min at room temperature in 15 µL of reaction buffer containing 25 µM ATP, 0.5 mg mL^{-1}

Myelin basic protein (Upstate, http://www.upstate.com), and 1 µCi γ^{32}P-ATP (Hartmann Analysis, http://www.hartmann-analysis.de). The reaction was stopped by the addition of SDS-PAGE sample buffer. The phosphorylated MBP was visualized by autoradiography after being resolved on a 15% SDS-polyacrylamid gel.

2. 2. 13 In Gel Kinase assay

Extracts containing 20 µg of total protein was loaded on 10 % (w/v) polyacrylamide gels embedded with 0.3 mg mL^{-1} Myelin basic protein (MBP, Upstate, http://www.upstate.com) in the separating gels as substrate for the kinase. After electrophoresis SDS was removed by washing the gel with washing buffer three times for 30 min each at RT. The kinases were allowed to renature in renaturation buffer at 4 °C overnight with three changes of buffer. The gel was then incubated at RT in 20 mL of reaction buffer for 60 min. The reaction was stoped by transferring the gel into washing buffer. The unincorporated γ^{32}P-ATP was removed by washing with the same buffer for 1 h with two changes. Activities were visualized by autoradiography and by a phosphor imager (Cyclone, Phosphor Storage Systems, Madison, WI).

2. 2. 14 Chromatographic Separations

Ground cells of a 50 mL of culture were used to prepare the crude extract with 10 mL of buffer A. After 30 min of centrifugation at 100,000g, supernatant containing 10 mg of total protein was loaded on the strong anion-exchange column Resource Q (6 mL, Pharmacia) equilibrated with buffer A. After wishing with 30 mL of buffer A, the column was eluted with 80 mL linear gradient of 0 to 500 mM NaCl in buffer A.

Fractions were analyzed by in-solution assay or in-gel kinase assay. Relative activity was calculated as described previously (Link *et al*., 2002a).

Running method

Break point	Conc % B	Flow	Fract	BufferV	InjectV	Comment
0	0	10 mL min^{-1}	0	Pos1	Load	Equilibration
30	0	5 mL min^{-1}	0	Pos1	Inject	Sample apply
50	0	10 mL min^{-1}	0	Pos1	Load	Elution delay/ wash
80	0	2 mL min^{-1}	2 mL	Pos1	Load	Elution and gradient start
160	50	10 mL min^{-1}	2 mL	Pos1	Load	Column wash
189.9	50	40 mL min^{-1}	0	Pos1	Load	End column wash
190	0	10 mL min^{-1}	0	Pos1	Waste	Priming
220	0	10 mL min^{-1}	0	Pos1	Load	Re-equilibration
230	0	10 mL min^{-1}	0	Pos1	Load	End method

2. 2. 15 Inhibitor studies

The inhibitors Bohemine, Olomoucine, Roscovitine, A.2.1.1, A.2.1.11, A.2.1.24, A.2.1.27, A.2.1.31, B.2.1.H, D.2.1.4, A.1.1.34, A.3a.1.1, A.2.C.6, A.2.C.Cl, A.1.2.1, A.2.2.1, A.2.3.6, A.2.P.25, B.2.12.H, A.H.8.Cl, and A.H.4.1 were synthesized (Havlicek *et al*., 1997) and SB203580, and ML3403 were synthesized by Dr. Laufer (Department of pharmaceutical and medical chemistry, Eberhard-Karls-university, Tuebingen, Germany). 1mg mL^{-1} solution in DMSO was diluted in water to final concentration of 10 µM in the in-solution kinase assay. Relative activity was calculated as described previously (Link *et al*., 2002a).

2. 2. 16 Transient expression in field garlic epidermal cells

Cells in the epidermal layer of field garlic (*Allium oleraceum*) bulbs were transformed

using biolistic bombardment essentially as described (Varagona *et al*., 1992). 50 mg of tungsten particles was suspended in 100 µL of sterile water. 20 µg of plasmid DNA were added, followed by the addition of 100 µL $CaCl_2$ (2.5 M), and 40 µL spermdine (100 mM in H_2O). After 3min vortex, 600 uL of EtOH was added. After incubation at -20°C for 30 min, the particles were washed with 100 µL of sterile water, and resuspended in 100 µL of sterile water. Tungsten particles coated by 10 µg of plasmid DNA (GFP, and GFP:*AtPDP1*) were briefly vortexes and bombarded onto layer of field garlic (Vacuum : -0.8 bar, Helium pressure : 9.0 bar). After bombardment, field garlic cell layers were incubated for 48 hr at 22°C in complete darkness. GFP fluorescence was visualized using laser scanning microscope system.

2. 2. 17 Histochemical detection of H_2O_2 and cell death

Detection of H_2O_2 was by endogenous peroxidase-dependent in situ histochemical staining using 3,3-diaminobenzidine (DAB) in a protocol modified from Samuel and Ellis (2002). Leaves infiltrated with AtPDP1 overexpression construct were vaccum-infiltrated with 1mg mL^{-1} of DAB solution, pH 3.8. Infiltrated leaves were placed under humidity and darkness condition until brown precipitation was observed (5 to 6 h) and then cleared in 90 % ethanol at 70°C until complete removal of chlorophyll. Dead cells were stained with 1 % trypan blue. Leaves were cleared by boiling in 30 % KOH for 5 min and stained in 1 % trypan blue for 10 min then cleared in 90 % ethanol at 70°C until complete removal of chlorophyll. Stained samples were mounted in 60 % glycerol and examined using a light microscope.

2. 2. 18 Preparation of *Pseudomonas syringae* pv. *tabaci* labeled with GFP

Pseudomonas syringae pv. *tabaci* competent cells were prepared according to the method described in Choi *et al*., (2006). Overnight cultured cells in LB medium were harvested by centrifugation at room temperature for 2 min at 16000 x g. The cell pellet was washed two times with sterilized water, and resuspended in sterilized water to 10^9colony-forming units (C.F.U) mL^{-1}. 500 ng of plasmid pPNptGreen (Sabaratnam and Beattie, 2003) was mixed with 50 µL of electrocompetent cells. After applying a pulse (setting: 2500 volt, and 4.8-5.0 sec on an Electoporator 2510; Eppendorf), 500 µL of LB medium was added. 2 hours after incubation at 28°C with shaking, the cells were harvested by centrifugation, resuspended in 100 µL LB medium, and then plated on LB medium containing 50 mg L^{-1} kanamycin. Positive colonies were screened under UV light. 10^5 C.F.U mL^{-1} of labeled *Pseudomonas syringae* pv. *tabaci* infiltrated into transgenic tobacco plants. 7 days after inoculation of labeled *Pseudomonas syringae* pv. *tabaci*, GFP fluorescence was observed with UV microscope system.

2. 2. 19 Bacterial growth assays

Bacterial strains used in this study were *Pseudomonas syringae* pv. *tabaci* (virulent strain). Bacteria were growth at 28°C on King's B medium containing appropriate antibiotics for selection. Syringe inoculation and bacterial growth in planta were performed as described previously (Zipfel *et al*., 2004). Bacteria were inoculated into 10 ml King's B medium with antibiotics and grown overnight at 28°C, collected by centrifugation and resuspended in 10 mM $MgCl_2$ to 10^5 colony-forming units (C.F.U)mL^{-1}, and infiltrated into leaves with 1 mL needleless syringe. Leaf discs were

harvested and ground in 10 mM $MgCl_2$. After grinding of leaf discs, the samples were diluted 1:100 with 10 mM $MgCl_2$, plated on LB medium containing antibiotics. 2 days after incubation at 28°C, the colony-forming units were counted.

2. 2. 20 Imaging of chlorophyll fluorescence parameters

The effective photosystem II quantum yield Y (II) was determined according to the method described in Bonfig *et al*., (2006). 5 days after treatment salt stress, Y(II) was analyzed using an Imaging–PAM chlorophyll Fluorometer (Model IMAG-K, Walz, Effeltrich). The area was individually marked by a circle for every leaf, which served as area of interest (AOI) over which all pixel values for the various fluorescence parameters were averaged.

3. Results

3. 1 Tomato mitogen activated protein kinases regulate the expression of extracellular invertase Lin6 in response to stress related stimuli

In plants, various members of the MAP kinase signal transduction pathway have been identified and linked to the transduction of diverse stimuli, including responsiveness to pathogens, wounding and other abiotic stresses. In recent years, evidence has accumulated that extracellular invertases are not only important to mediate carbohydrate supply to sink tissues, but that they are co-ordinately induced with defense related genes (Roitsch *et al*., 2003). The use of kinase inhibitor and protein phosphatase inhibitors suggested that phosphorylation reactions are also involved in the regulation of extracellular invertases by stress related stimuli (Ehness *et al*., 1997). Since MAP kinases have been shown to be key signaling components to mediate defense reactions, it has been analyzed whether they are also involved in the signal transduction pathways to mediate the co-ordinate regulation of sink gene expression (Roitsch *et al*., 2003) and defense reactions. Thus we have cloned *LpMPK2* and *LpMPK3* from *Lycopersicon peruvianum* and established with complementing experimental approaches a relation between the fast and transient activation of MAP kinases and induction of extracellular invertase *Lin6*.

3. 1. 1 Cloning of *LpMPK2* and *LpMPK3* from *Lycopersicon peruvianum*

To demonstrate the relationship between MAP kinase activation and *Lin6* expression, we isolated a tomato homolog of *LeMPK2* and *LeMPK3* named *LpMPK2* and

LpMPK3, respectively from *L*. *peruvianum*. Gene specific primers were designed against the *LeMPK2* sequences (Accession number: AY261513.1) to clone *LpMPK2* while degenerate primers based on conserved amino acid sequences in the N-terminal part of TC85947 and C-terminal part of TC87269 were used to amplify full length cDNA of *LpMPK3* obtained from total RNA prepared from tomato cells, which are treated with the fungal elicitor E-Fol, derived from *Fusarium oxysporum lycopersici*.

Both *LpMPK2* (Accession number: EU887292) and *LpMPK3* (Accession number: EU178755) contain the 11 subdomains, conserved among all MAP Kinase families and possesses a dual phosphorylation motif TEY common to most plant MAP kinases. Phylogenetic analysis of representative plant MAP Kinase based on amino acid sequence alignment reveals that LpMPK2 and LpMPK3 belongs to the A subgroup (Figure 5). This subgroup represented by *AtMPK3* and *AtMPK6* of *Arabidopsis*, *WIPK* and *SIPK* of tobacco, and *MsMMK4* of Alfalfa appears to be activated by a variety of stresses. This subgroup is also characterized by increases in their mRNA levels in response to the same stresses.

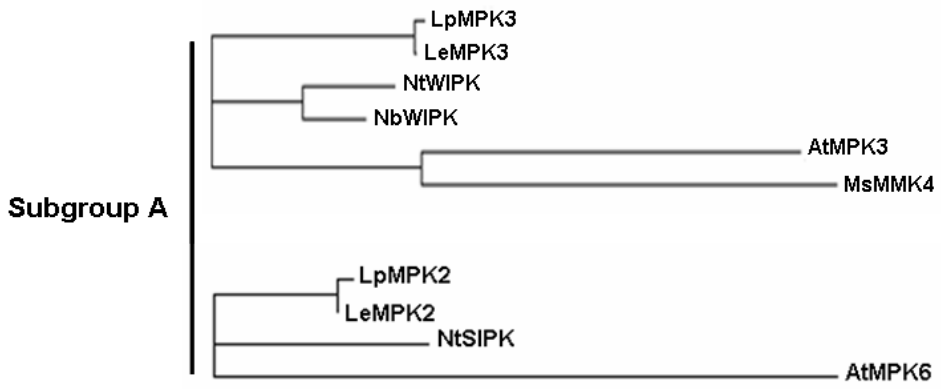


Figure 5. A phylogenetic tree based on amino acid sequence alignment of MAP kinases from other higher plants. A phylogenetic tree shows the relationship among the A group of plant MAP kinases (MAPK group, 2001). The tree was created by using the ClustalW (www.ebi.ac.kr/Tools/clustalw2/index.html).

3. 1. 2 Transcript of MAP kinase *LpMPK3* and extracellular invertase *Lin6* are sequentially induced by different stress related stimuli

The MAP kinase activation and *Lin6* gene expression were shown to be induced by various stresses such as pathogen infection and wounding. In addition, protein kinase inhibitor, staurosporine, inhibited the induction of extracellular invertase expression by fungal elicitor and protein phosphatase inhibitor indicating the involvement of kinases in the upstream signaling pathway (Ehness *et al*., 1997). To verify this correlation, we analyzed the level of mRNA for *LpMPK3* and *Lin6* in response to stress related stimuli. It was observed that the treatment of photoautotrophic cell culture of tomato with the fungal elicitor E-Fol, the endogenous, plant derived elicitor polygalacturonic acid (PGA) and KCl resulted in the upreguation of transcripts of both *LpMPK3* and *Lin6* (Figure 6). The accumulation of *LpMPK3* transcript was fast and transient and was highest at 30 min after the addition of the stimuli, while the delayed accumulation *Lin6* transcript was always observed after the peak of *LpMPK3* and the maximum induction was found at six hours after the stress treatment. The sequential induction supports a function of *LpMPK3* in the stress related expression of *Lin6* and that expression and activation of *LpMPK3* might be required for the induction of *Lin6* expression.

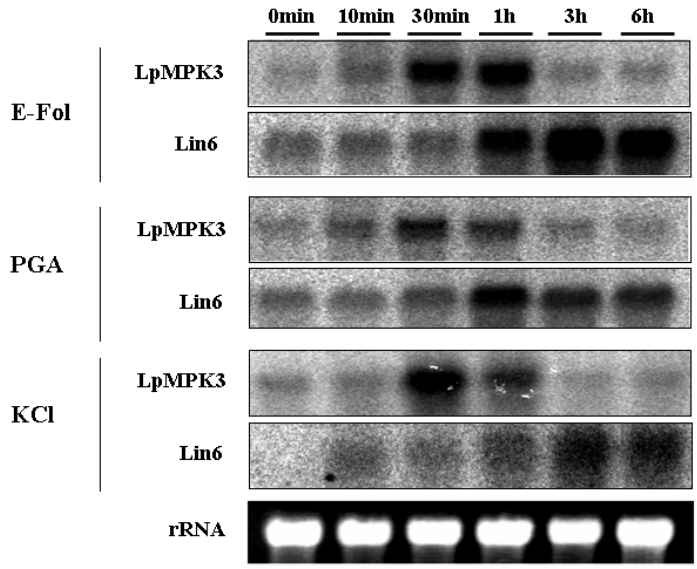


Figure 6. Regulation of mRNAs for *LpMPK3* and *Lin 6*. Thirty micrograms of total RNA was separated on formaldehyde agarose gels, blotted onto nitrocellulose, and probed with random primer labeled cDNA fragments. Equal loading of RNA was confirmed by ehtidium bromide staining of the rRNA. According to Hoffmann (2003), with additions.

3. 1. 3 Fast and transient activation of LpMPK2 and LpMPK3 by wounding, E-Fol, PGA, and KCl in tobacco

To analyze the activation of kinase activity of LpMPK3 and LpMPK2, we transiently transformed tobacco leaves with a modified version of *LpMPK3* carrying a triple HA-tag (*LpMPK3*-HA) and *LpMPK2* carrying His-tag (*LpMPK2*-His) in C-terminals. *Agrobacterium* cells carrying the *LpMPK3*-HA and *LpMPK2-His* construct were infiltrated into the leaves of 4- to 8-week old tobacco plants. Transgene expression was induced by wounding. Activated LpMPK3 and LpMPK2 were immuno-precipitated with HA11 and His antibodies, respectively, and the activity was analyzed in an in-solution assay. Wounding induced activation of both LpMPK3 (Figure 7) and LpMPK2 (Figure 8) within 3 min. Maximum activation of LpMPK3 was observed at 24 min after wounding. We also examined the activation of both the MAP kinases by

elicitors, E-Fol and PGA, and salt stress, KCl. Transiently transformed leaves of tobacco were infiltrated with 100 µg mL^{-1} E-Fol, 100 µg mL^{-1} PGA and 50 mM KCl. The protein extracts were immuno-precipitated before analyzing the activity in an in-solution assay. A fast and strong activation of LpMPK3-HA and LpMPK2-His could be observed by all stimuli (Figure 7 and 8). Though the application of PGA activated LpMPK3 within 3 min, the activation of LpMPK2 could be observed only after 12 min. These data show that both LpMPK3 and LpMPK2 are activated by all stress related stimuli tested, elicitors, wounding and osmotic stress, and thus are part of the plant defense response.

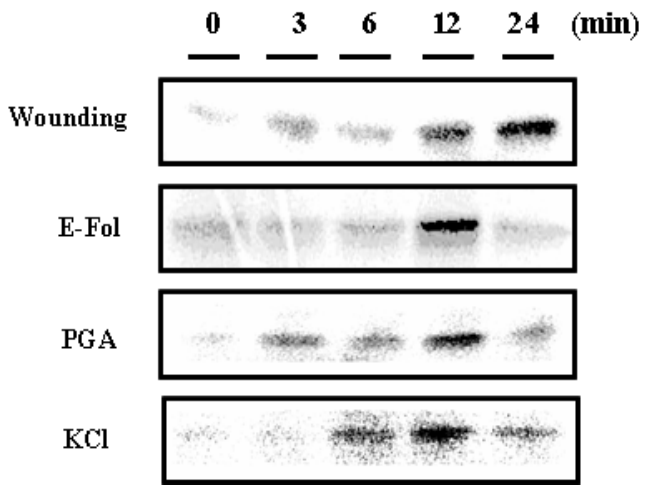


Figure 7. Time-course analysis of LpMPK3 activity induced by wounding, E-Fol, PGA and KCl treatments. The protein kinase activity of LpMPK3-HA was analyzed by immunoprecipitation HA11 antibody coupled with an in-solution assay. Phosphorylated MBP was visualized by autoradiography.

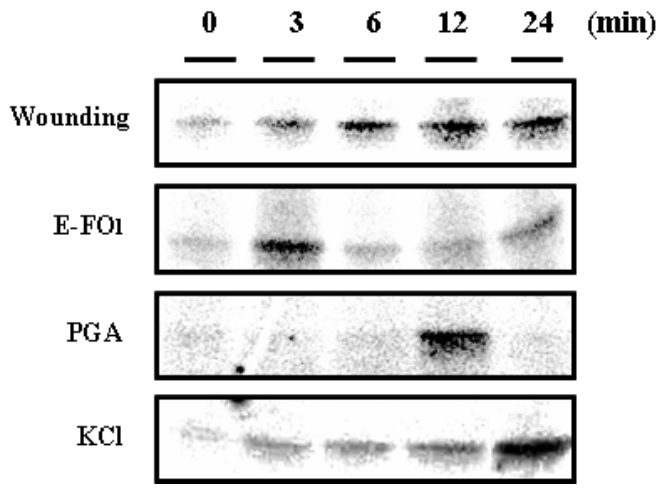


Figure 8. Time-course analysis of LpMPK2 activity induced by wounding, E-Fol, PGA and KCl treatments. The protein kinase activity of LpMPK2-His was analyzed by immunoprecipitation His antibody coupled with an in-solution assay. Phosphorylated MBP was visualized by autoradiography.

3. 1. 4 Induction of the Lin6 promoter by stress-related stimuli seems to be mediated by MAP kinases

To assess the role of *Lin6*, in stress signal transduction by MAP kinases, we studied the relationship between Lin6 and LpMPK3/LpMPK2 activation. For this purpose, we expressed *LpMPK3*-HA and *LpMPK2*-His under the control of a CaMV35S promoter. As transformation control for *LpMPK3*-HA, an RNAi construct of LpMPK3, while for *LpMPK2* a corresponding vector without the transgene was used. *Agrobacterium* cells carrying either *LpMPK3*/*LpMPK2* or the transformation control constructs were infiltrated into the leaves of transgenic tobacco plants expressing a *Lin6* promoter::GUS fusion. Two days after infiltration, the plants were treated either by wounding or by infiltration with E-Fol, PGA and KCl, and analyzed for transgene expression. Non-inoculated transgenic tobacco leaves harbouring the GUS reporter gene driven by *Lin6* promoter were taken as control. Tobacco leaf protein extracts were analyzed 2 h, 24 h and 48 h after the treatment with the different stress related

stimuli for the activation of the *Lin6* promoter by the fluorometric GUS reporter gene assay. A strongly increased GUS activity was detected in all samples derived from leaves expressing *LpMPK3*-HA after the various stress treatments, although stress type dependent differences in the time course and extend were evident (Figure 9). In contrast, low GUS activities were detected both in all transformation control (*LpMPK3*-RNAi) samples and samples from leaves that were not transformed by agroinfection. Interestingly, samples expressing *LpMPK2*-His also showed higher GUS activities with all stress treatments, but in this case, samples expressing transformation control also showed higher activities compared to non-transformed samples (Figure 10). Since the absolute values differed between individual experiments, representative results from the independent experiments for the various stimuli are shown in Figure 8 and 9. The increase of the *Lin6* promoter driven GUS activity in response to wounding or infiltration of E-Fol, PGA and KCl seems to be dependent on the expression of both *LpMPK3*-HA and *LpMPK2*-His, which were shown before to be activated by the same set of stimuli (see above). Apparently, since there was no increase in the Lin6 promoter activity in transformation control *LpMPK3*-RNAi while increase in the vector control for LpMPK2, indicates that LpMPK3 might be involved in the expression of this important enzyme of primary metabolism. The induction of the Lin6 promoter by wound was sustained in *LpMPK3*-HA expressing sample, the induction by E-Fol, PGA and KCl was transient. Whereas in case of *LpMPK2*-His expressing samples, the induction of GUS activity was transient during wounding stress while it was sustained during other stress treatments. The correlation between the expression of Lin6 promoter and the activation of LpMPK3 and/or LpMPK2 by different stress related stimuli support that the induction of the Lin6 promoter requires the expression of MAP kinases.

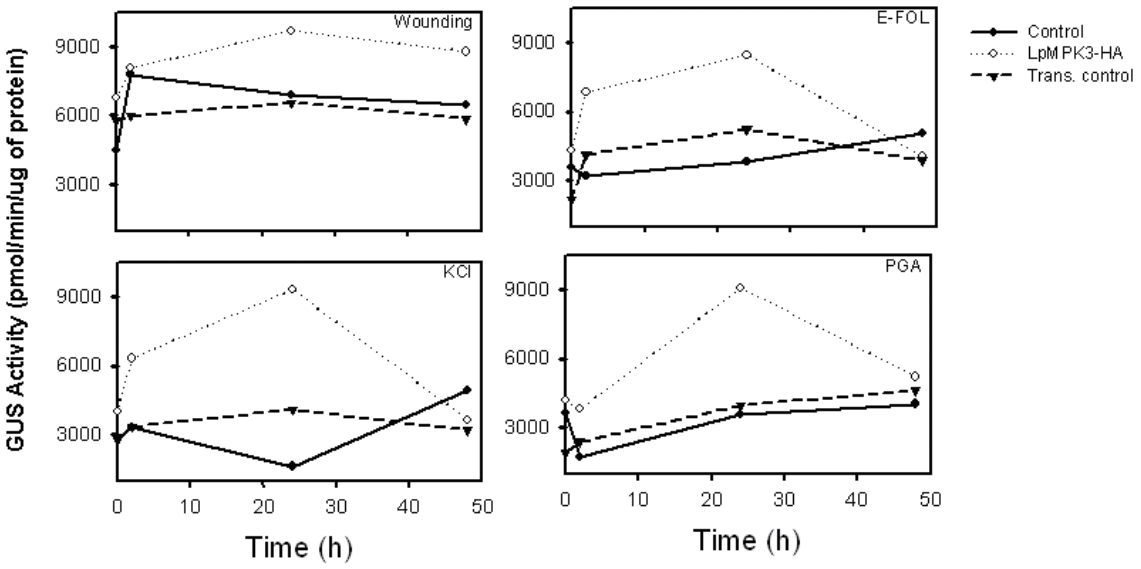


Figure 9. The activation of LpMPK3 leads to expression of Lin 6 promoter after treatment of stimuli. Experiments were repeated three times with similar results and representative data is presented.

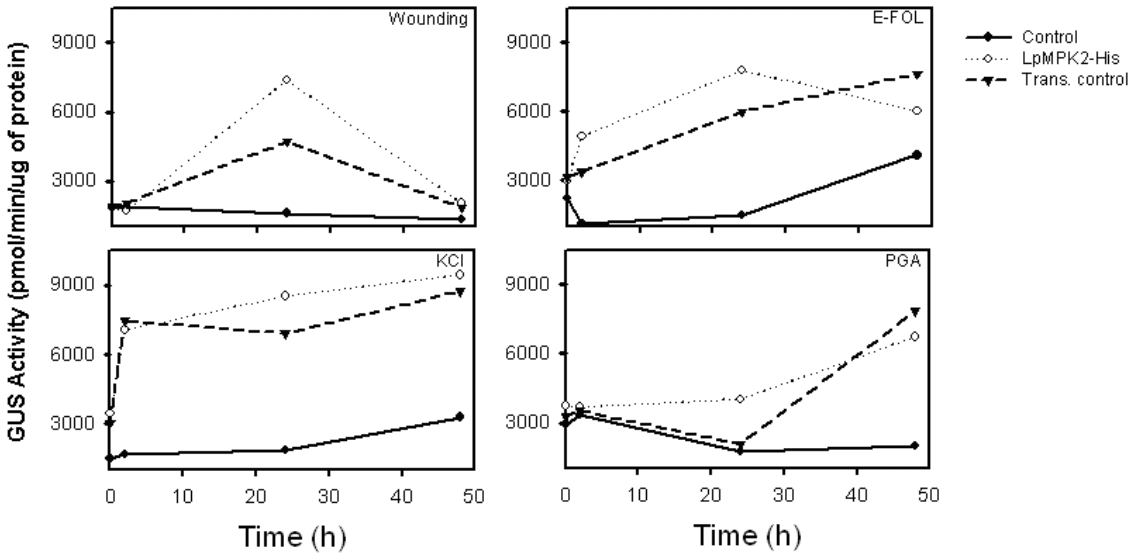


Figure 10. The activation of LpMPK2 leads to expression of Lin 6 promoter after treatment of stimuli. Experiments were repeated three times with similar results and representative data is presented.

3. 2 Glucose and Oxylipins induced the activation of AtMPK4 and/or AtMPK6

3. 2. 1 MAP kinases are activated by glucose.

Plant sugars not only serve as metabolic resources and structural constituents of cells but also have hormone-like regulatory activities. Sugars modulate nearly all fundamental processes throughout the entire life cycle of plants, including embryogenesis, germination, growth, development, reproduction, senescence, and responses to diseases and environmental stimuli (Roitsch, 1999; Smeekens, 2000; Rolland *et al*., 2006). Several studies indicate a critical role of protein phosphorylation in sugar dependent signal transduction pathways (Ohto and Nakamura, 1995; Sinha *et al*., 2002; Roitsch *et al*., 2003). However, the involvement of MAP kinase cascades by sugar signaling cannot be ruled out.

Plant cell suspension cultures are used as one of systems for studying plant metabolism and signal transduction against stimuli. In particular, photoautotrophic cultures of *C. rubrum* (Ehness et al., 1997) and tomato (Sinha et al., 2002) proved to be useful to address various aspects of source-sink regulation in response to sugar and stress-related stimuli, and photoautotrophic cultures of *Arabidopsis thaliana* has been established by Roitsch Lab. *Arabidopsis* is being used as model research plant as the complete genome has been sequenced and also various knock-out mutants are available. Here, we have reported for the first time the photoautotrophic cell culture of model plant system *Arabidopsis thaliana* which can be used as model research material to study very fast events like signal transduction pathways including activation of MAP kinases.

Initially we tested glucose to characterize the activation of MAP kinases in response

to metabolizable sugar signals using the photoautotrophic cell culture of *Arabidopsis*. An in-gel kinase assay was used to determine whether the glucose induced the activation of MAP kinase. *Arabidopsis* photoautotrophic cultured cells were treated with 50 mM glucose or mannitol, and protein extracts were prepared. Protein kinase activity was analysed by in-gel kinase assay using myelin basic protein (MBP). As shown in Figure 11, glucose induced the activation of protein kinases of 47 and 44 kDa. The activity of MAP kinase peaked at 30 min and returned to a basal level within 160 min after treatment with glucose. In contrast, 50 mM mannitol did not induce the activation of MAP kinases.

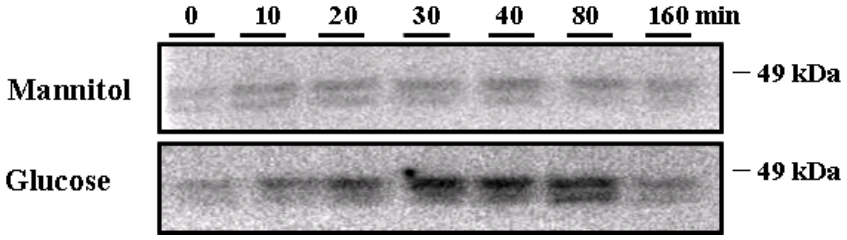


Figure 11. MAP Kinase activation by treatment with glucose or mannitol. Suspension cultures were treated with 50 mM mannitol or glucose. Sample were harvested at the indicated times for protein preparation. MAP kinase activity was determined by an in-gel kinase assay with MBP as a substrate.

3. 2. 2 MAP kinases are activated by OPDA, PPA_1 and PGA_1, but not by other oxylipins

In plants, like in animals, the oxylipin pathway regulates many biological processes, which include responses to development and environmental stimuli. Oxylipins are derived from the oxidation of α-linolenic (18: 3) and linoleic acids (18: 2) in plants, while they are derived from arachidonic acid (20: 4) in animals (Chehab *et al*., 2007). However, little is known about how the signaling of oxylipins is perceived and

transduced in plant cells. It has been shown that PPA_1 and PPB_1 (A_1- and B_1-phytoprostanes) activate MAP kinase in tomato cell cultures (Thoma *et al*., 2003). In addition, the treatment of photoautotrophic cell culture of tomato with the MeJA and PPB_1 resulted in the upreguation of transcripts of *Lin6*, whereas PPA_1 did not induce the expression of *Lin6* (Thoma *et al*., 2003). These findings suggest that oxylipins have crucial impact on cell signaling and primary metabolism, and could be extended to *Arabidopsis* cells for the identification of oxylipins induced signal transduction pathways.

In this study, the effect of oxylipins on MAP kinase activation was tested in *Arabidopsis* photoautotrophic cultured cells treated with a solution of OPDA (75 µM), MeJA (75 µM), PPA_1 (75 µM), Hexenal (75 µM), Hydroxy nonenal (75 µM) and PGA_1 (100 µM). As shown in Figure 12, OPDA and PPA_1 resulted in strong activation of 47 kDa kinase, wherese MeJA, Hexenal, or Hydroxy nonenal did not induced MAP kinase activity.

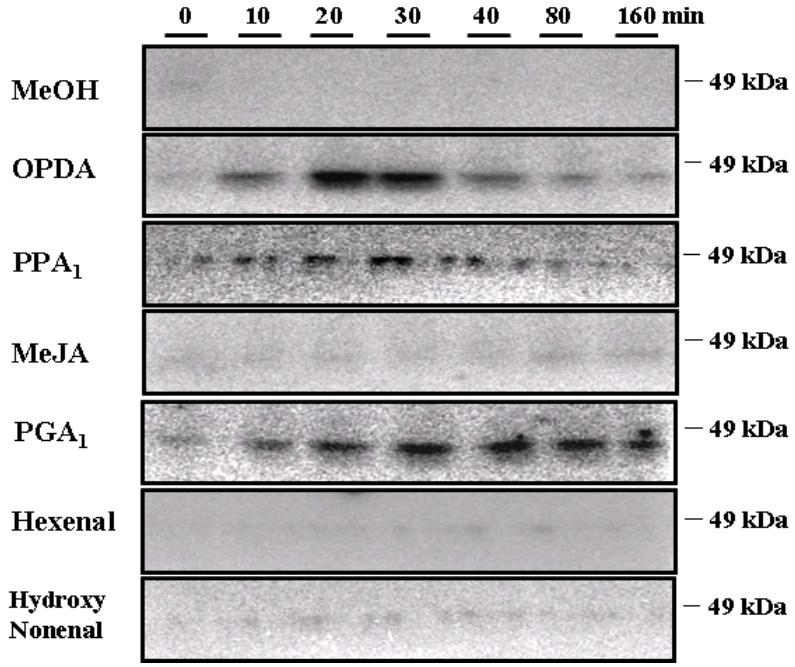


Figure 12. MAP Kinase activation by treatment with oxylipins. Suspension cultures were treated with 75µM OPDA, MeJA, PPA_1, Hexenal or Hydroxy nonenal, and 100µM PGA_1. MAP kinase activity was determined by an in-gel kinase assay with MBP as a substrate.

Interestingly, PGA1 which is produced by animals, but not by plants also strongly induced the activation of MAP kinase within a few minutes and this activity peaked within 40 min. This data indicates that the effect of oxylipins on MAP kinase activation may be mediated by the structure-specificity, and thus because of their physiochemical properties, their biological properties are not simple.

3. 2. 3 Purification of the glucose or oxylipins-induced MAP kinases on anion exchange chromatography

The glucose or oxylipins-induced MAP kinase was partially purified by ultracentrifugation and separation on a Resource Q anion exchange column. As shown in Figure 13. by an in-gel kinase assay, we were able to separate a highly enriched MBP phosphorylating activity after OPDA, PPA_1 or PGA_1 treatments. Interestingly, two peaks with MBP phosphorylating activity were separated after glucose treatment. The active fractions eluting from the Resource Q anion exchange column were pooled and used for characterization of these MAP kinases. The partially purified protein tested for their ability to phosphorylate other substrates like histone III-SS and caseine along with MBP. Only MBP was proved to be a good phosphate acceptor, whereas histone III-SS and caseine were a poor substrate for the partially purified kinase (Figure 14). This result suggests that the pooled fractions contain only a single kinase activity.

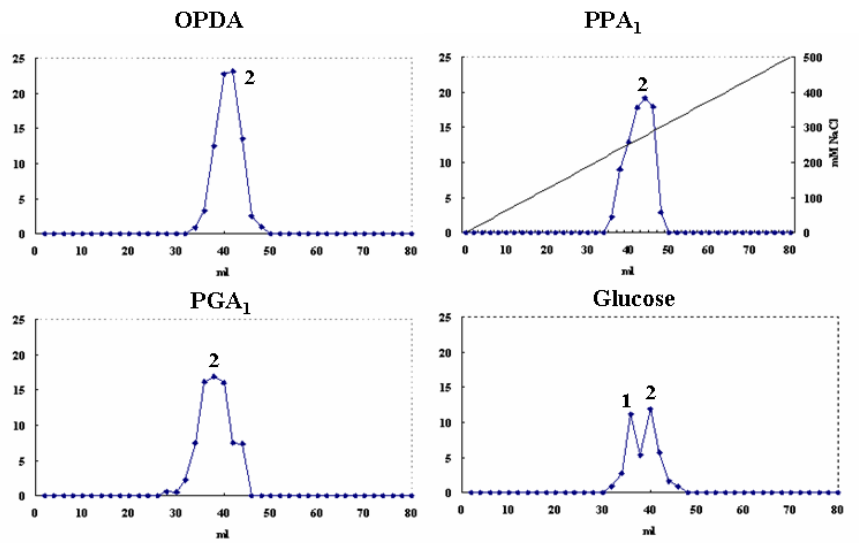


Figure 13. Separation of cell extracts by anion-exchange chromatography. Extracts from cells treated with the indicated stimuli were separated and analyzed by in-gel kinase assay. Activity was quantified by phosphor imager and diagrammed as x-fold activity of untreated cells. The salt-gradient is exemplary depicted in the PPA_1 diagram.

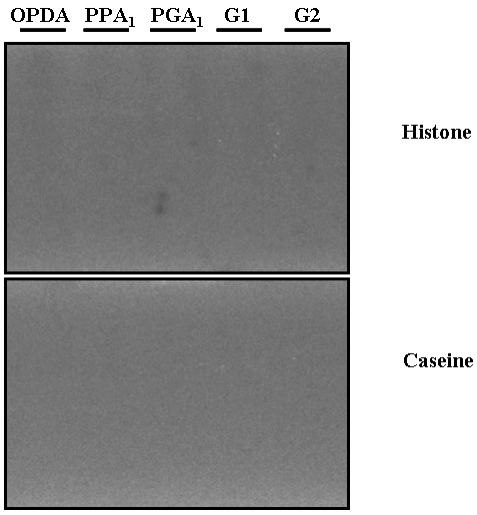


Figure 14. Separated protein kinases do not phosphorylate Histone and Caseine. Kinase activities in separated peak fraction were determined by in-gel kinase assay with histone III-SS or caseine as a substrate.

3. 2. 4 Immunoprecipitation with specific *Arabidopsis* MAP kinase antibodies.

The use of MBP as a preferred substrate suggested that the partially purified protein might be a MAP kinase. To characterize this protein, we employed the AtMPK3,

AtMPK4, or AtMPK6-specific antibody in immuno complex kinase assay. Figure 15 shows that the MBP phosphorylating activity of peak fraction from *Arabidopsis* cells treated with OPDA, PPA_1 or PGA_1 was precipitated by AtMPK6 antibody. Also, Glucose 2 (Second peak fraction from glucose treated cells) was precipitated by AtMPK6 antibody. Interestingly, Glucose 1 (first peak fraction from glucose treated cells) was precipitated by AtMPK4 and AtMPK6 antibodies. No activity was precipitated by AtMPK3 antibody. These results suggest that OPDA, PPA_1 and PGA_1 induce the activation of AtMPK6, and 47 and 44 kDa protein kinases induced by glucose are AtMPK6 and AtMPK4.

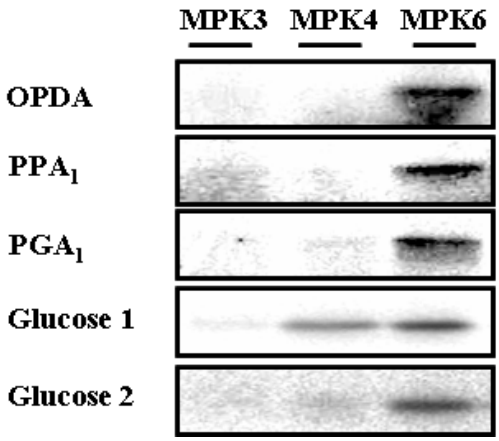


Figure 15. AtMPK4 and AtMPK6 are activated by oxylipins and glucose. Peak fractions were analyzed by immunoprecipitation with the antibodies AtMPK3, AtMPK4, or AtMPK6 and subsequent in-solution assay.

3. 3 The elevated levels of CO_2 induce the activation of Mitogen-activated kinases in tobacco

In many case, photosynthesis is limited by the availability of CO_2 via the process of diffusion from atmosphere to site of photosynthetic CO_2 fixation (Uehleim *et al*., 2008). It has been shown that the source-sink imbalance is mediated by the elevated level

of CO_2, and results in the down-regulation of the small subunit of Rubisco (Gesch *et al*., 1998). Glucose levels are often high in plants grown at elevated CO_2 or fed sugar where Rubsico expression is repressed (van Oostn and Besford, 1996). This potentially provides the importance of CO_2 in soure-sink balance in higher plants.

MAP kinases play important signaling roles in plant's response to environmental stresses. They are rapidly and transiently activated by different stress-related stimuli (Zhang *et al*., 2006). In addition, the stomata did not response to elevated levels of CO_2 in NtMPK4-silenced plant. However, the constitutively active SIPKK ($SIPKK^{EE}$) plant, which act upstream of NtMPK4, has shown no difference in the CO_2 responses compared with wild type plant (Marten *et al*., 2008). These suggest that the activation of MAP kinases act as a positive regulator of stomatal movement and other MAP kinases may be activated in parallel with NtMPK4 for stomatal movement. However, to our knowledge, thus far, there is no evidence about the activation of MAP kinase cascades by CO_2.

To investigate if any MAP kinase could be activated by elevated levels of CO_2 in plants, we treated tobacco leaves with 700 μL L^{-1} of CO_2 (2 fold increased level compared to atmospheric CO_2) for various times. Protein extracts were prepared and kinase activity was determined by an in-gel kinase assay with myelin basic protein (MBP). As shown in Figure 16A, elevated levels of CO_2 induced the fast and transient activity of MAP kinases, two protein kinases with molecular masses of about 48 and 45 kDa. Maximum activity was reached at 2 min, and the activity fell to background levels within 5 min.

To compare the strength of CO_2 stimuli in MAP kinase signaling, we induced the activation of MAP kinase from tobacco by treatment with 100 μg L^{-1} E-Fol. The activity of MAP kinase was analyzed by in gel kinase assay, and diagrammed as x-fold activity at time zero set equal to 1 (Figure 16B). CO_2 induced 1.5 fold increase in

kinase activity, whereas E-Fol induced approximately 8 fold increase in kinase activity. These data show that 48 and 45 kDa protein kinases are slightly activated by elevated levels of CO_2 compared with the stimuli induced MAP kinase activity.

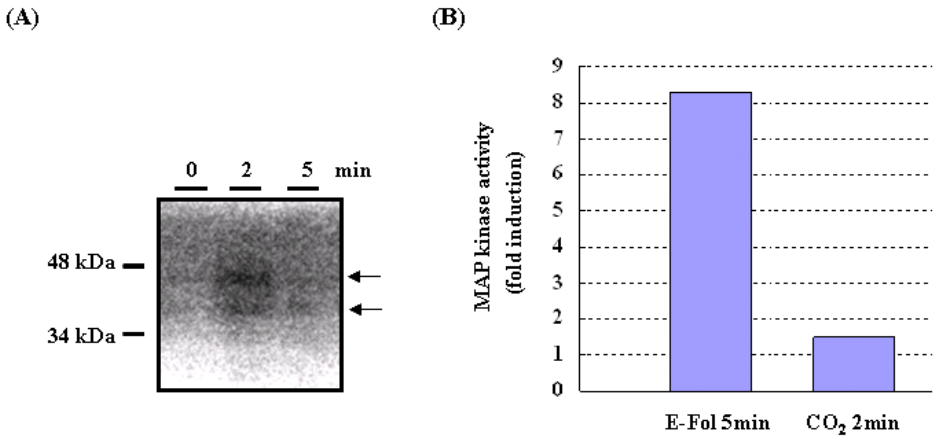


Figure 16. The elevated levels of CO_2 induced the rapid and transient activation of kinases. Leaves of 4 week-old tobacco plants were treated with 700 µL L^{-1} of CO_2 (A) or infiltrated with 100 µg mL^{-1} of E-Fol (B). Kinase activity was monitored with an in-gel assay using MBP as the substrate. (B) The activity of MAP kinase is shown as the fold induction of kinase activity, with the level of activity at time zero set equal to 1. Molecular size markers are indicated at right. The positions of activated kinases are marked by arrows.

MAP kinases are enzymatically activated when threonine and tyrosine residues within the TXY motif in subdomain VIII are phosphorylated by MAPK kinase (MAPK group, 2002). To further support that these MBP-phosphorylating kinases activated by elevated levels of CO_2 belong to the MAP kinase family, we immunoprecipitated the extract with the phosphotyrosine- specific mono cloned antibody 4G10. Thereafter, each immunopreciptated MAP kinase was tested for activation by in-solution assay rather than in-gel kinase assay, because in-solution assay is more sensitive method than in-gel kinase assay for detecting low level activity. The kinases activity transiently increased at 2 min and declined to the basal level within 10 min (Figure 17A). Though a basal level of constitutive activation of MAPK was also

observed in control samples.

To further characterize MAP kinases activated by CO_2, extracts from treated samples were subjected to an immune complex kinase assay using anti-AtMPK6 or anti-NtMPK4. The sequence of anti-AtMPK6 is highly conserved in the *Nicotiana tabacum* ortholog SIPK. Figure 17B and 17C show that the MBP-phosphorylating kinase activity was precipitated by AtMPK6 and NtMPK4 antibody. These data suggest that high level of CO_2 induce the activation of SIPK and NtMPK4 in tobacco.

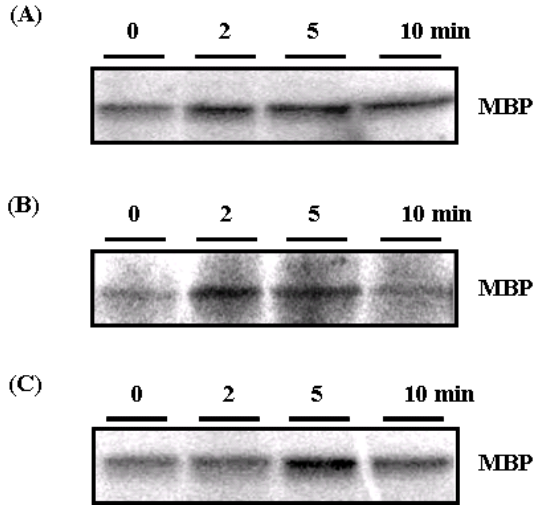


Figure 17. SIPK and NtMPK4 are activated by elevated levels of CO_2. Leaves of 4 week-old tobacco plants were harvested at the indicated times after treatment of CO_2, and immune complex kinase assays were performed using 4G10 (A), anti-NtMPK4 (B), and anti-AtMPK6 (C) antibodies.

Purine analogue inhibitors, Roscovitine, Olomucine and Bohemine, two p38 inhibitors SB203580 and ML3403, and Staurosporine, a non-specific inhibitor of many serin/threonine protein kinases, were used to address their effect in vitro on the CO_2 induced protein kinase activity present in crude extracts prepared from tobacco leaf after 2 min treatment with elevated levels of CO_2. Figure 18 displays that these compounds act as an inhibitor of CO_2 induced protein kinase activity. 10 µM of Roscovitine, Olomucine or Bohemine resulted in 49 %, 38 % or 44 % inhibition

whereas SB203580 and ML3403 did not inhibit the CO_2 induced protein kinase activity. It has been shown the inhibitory effect of CDK inhibitors on the elicitor induced tomato MAP kinase and the MMK1 MAP kinase from alfalfa (Binarova *et al*., 1998; Link *et al*., 2002a). SB203580 is a typical inhibitor of the vicinal diaryl class and exhibit strong inhibitory activity against p38, but not against ERK2 or other serin/threonine kinases (Wang *et al*., 1998). In addition, ML3403 belongs to the second generation of diacylimidazole-type p38 inhibitor (Kammerer *et al*., 2007). No inhibitory effect of both compounds on CO_2 induced protein kinases activity indirectly suggests that these kinases have different structure compared with p38.

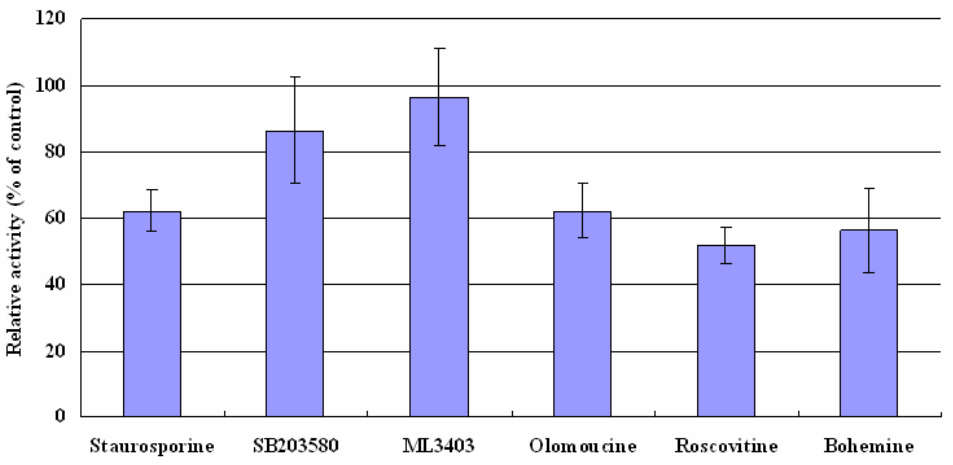


Figure 18. The activity of CO_2 induced protein kinase is inhibited by Cyclin dependent kinases inhibitors, but not p38 kinase inhibitors. ATP was present at 25 µM in all assays. The activity was quantified and is depicted as percentage of the activity without addition of inhibitor. SD of three experiments is depicted.

3. 4 Non-antioxidant function of tocopherol in plants: α-Tocopherol inhibits stress induced MAP kinase activation

In plants, tocopherols have been the subject of various studies that have emphasized their importance in the plant defense mechanism responding to photoinhibition and

photooxidative stress (Havaux *et al*., 2005), cold stress (Maeda *et al*., 2008), drought stress (Liu *at al*., 2008), and osmotic and salt stress (Abbasi *et al*., 2007). It has been shown that tocopherol deficiency affects the accumulation of sucrose, glucose and fructose, whereas starch level increased similarly in both *vte 2* and the wild type plants (Maeda *et al*., 2008), although the mechanism still remains an open question.

Since tocoperol has been shown to be the key compound to mediate oxidative stress defense reactions in plants, it has been analyzed whether tocopherol have non-antioxidant function which regulate the signal transduction pathway in animals (Ekstrand-Hammarstroem *et al*., 2006; Zingg, 2007). Taken together, these finding prompted us to investigate the non-antioxidant function of tocopherol on stimuli-induced MAP kinase which regulates the source/sink metabolism.

3. 4. 1 The activation of MAP kinase is induced by E-Fol and α-tocopherol phosphate, but not by α-tocopherol.

Initially we examined whether α-tocopherol induce activation of MAP kinases. BY2 cells were treated with 50 μM of α-tocopherol or 100 μg mL^{-1} of E-Fol for the activation control. Elicitor-treated cell cultures have been successfully used to study plant defense reactions involved in MAP kinase signal transductions. Our previous studies showed an elicitor preparation of the wilt-inducing fungus *F. oxysporum lycopersici*, referred to as E-Fol, resulted in strong MAP kinase activation in photoautotrophic cultures of tomato and *C. rubrum* (Link *et al*., 2002a; Ehness *et al*., 1997). MAP kinase activity was analyzed by in-solution assay using myelin basic protein (MBP) as an artificial substrate. E-Fol induced rapid and transient activation of protein kinases which can phosphorylate MBP. Maximum activity of protein kinases was reached at 10 min, whereas α-tocopherol did not induce the MBP-

phosphorylation activity (Figure 19A and C). Actually, α-tocopherol slightly activated protein kinases within 5 min, however, treatment of acetonitril as a control also slightly induced activation of protein kinases (data not shown). We also challenged α-tocopherol phosphate (TPh), a phosphoric acid ester of α-tocopherol, to investigate whether the action of α-tocopherol is mediated by its structure. Protein kinases are transiently activated 5 min after treatment with 50 μM of TPh and is found to be deactivated approximately 30 min after treatment (Figure 19C).

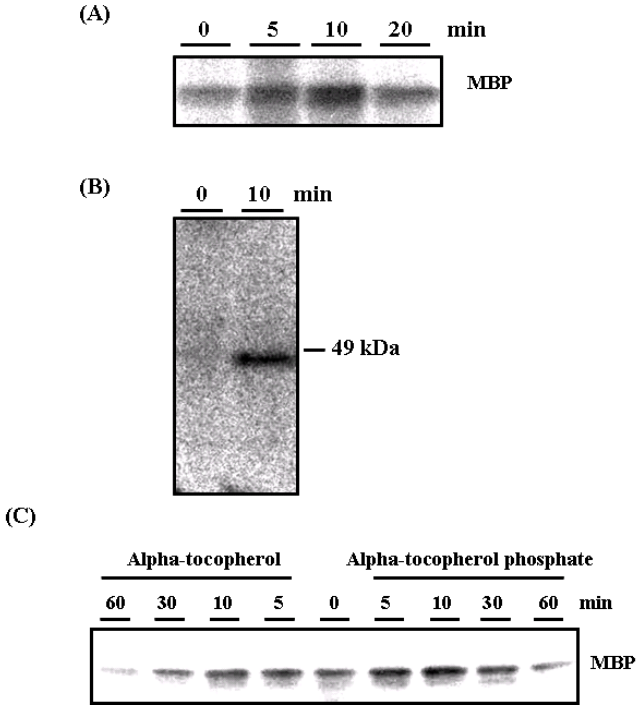


Figure 19. Rapid activation of MAP kinases by E-Fol but not by α-tocopherol. The protein kinase activity was analyzed by in solution assay. Phosphorylated MBP was visualized by autoradiography. BY2 cells were treated with 100 μg mL^{-1} of E-Fol (A), or 50 μM of α-tocopherol or α-tocopherol phosphate (C). After treatment of E-Fol for 10 min, the activation of MAP kinases was analyzed by an in-gel kinase assay (B).

To ensure whether MBP-phosphorylation activity is mediated by induction of MAP kinases activation, the E-Fol-treated cells were analyzed by in-gel kinase assay which involves de- and re-naturation cycles. The activation of MAP kinases was

strongly induced by E-Fol within 10 min (Figure 19B). These results suggest that the increased activity of MBP-phosphorylation by E-Fol is mediated by elevated level of MAP kinases activation. In addition, small modification of α-tocopherol caused the change of physiological function.

3. 4. 2 α-tocopherol inhibits the activation of elicitor- induced MAP kinases

To assess the role of α-tocopherol, in stress signal transduction by MAP kinases, we studied the relationship between α-tocopherol and stimuli-induced MAP kinases activation. For this purpose, we pre-incubated BY2 cells with 50 μM of α-tocopherol following different time course. After pre-incubation with α-tocopherol, cell cultures were challenged by E-Fol, and activation of MAP kinases was analyzed by in-solution assay and in-gel kinase assay. α-tocopherol inhibits activitation of protein kinases induced by E-Fol as shown in Figure 20A.

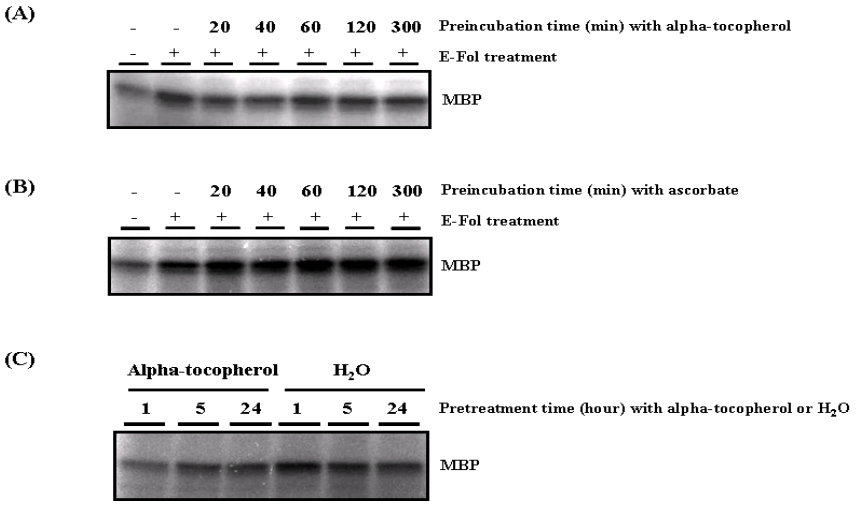


Figure 20. Effect of α-tocopherol on stimuli-induced MAP kinases. BY2 cells were pretreated with 50 μM α-tocopherol (A) and 50 μM ascorbate (B), and then treated with E-Fol for 10 min. The protein kinase activity was analyzed by in-solution assay. (C) The leaves of tobacco were infiltrated with 50 μM of α-tocopherol in H_2O. After 1, 5 or 24 hours of infiltration, the activation of MAP kinases was induced by wounding for 10 min. The protein kinase activity was analyzed by in-solution assay.

Pre-incubation of BY2 cells with α-tocopherol for 20 min slightly reduced MBP-phosphorylation induced by E-FoI as monitored by in-solution assay. However, pre-incubation with α-tocopherol for 40 min before the stimulation with E-FoI resulted in strong inhibition of MBP-phosphorylation activity. Interestingly, the inhibitory effect on MAP kinases activation was dependent on incubation time with α-tocopherol. When pre-incubation time was longer than 40 min, this inhibitory effect was reduced following the time course.

MAP kinases activity was also analyzed by in-gel kinase assay using crude extracts from BY2 cells treated with E-FoI, and BY2 cells which were stimulated with E-FoI after pre-incubation with α-tocopherol for 40 min (αtE). The base activity of MAP kinases was very low in BY2 cells (data not shown), while stimulation with E-FoI for 10 min showed strongly increased activity of MAP kinases. However, this indcution of strong activation of MAP kinases was reduced in BY2 cells pre-incubated with α-tocopherol (Figure 21A).

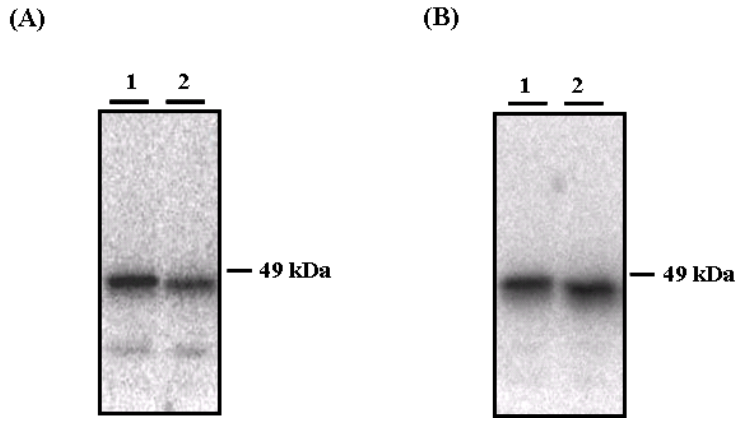


Figure 21. α-tocopherol down-regulates the upstream of MAP kinases. (A) The activation of MAP kinase was analyzed by in-gel kinase assay. 20 μg of crude extract from E-FoI treated cells (lane 1) and αtE (lane 2) was loaded on 10 % polyacryamide gel embed with MBP, phosphoryled MBP was visualized by autoradiography and by a phosphor imager. (B) The activity of protein phosphatases was analyzed by in-gel kinase assay. The crude extract from E-FoI treated cells was incubated with same amount of extraction buffer (lane 1) or αtE (lane 2) at RT for 20 min. After de- and renaturation cycles, phosphoryled MBP was visualized by autoradiography and by a phosphor imager.

To investigate whether inhibitory effect of α-tocopherol on MAP kinase activation is mediated by antioxidant function, we pre-incubated BY2 cells with 50 μM of ascorbate instead of α-tocopherol. After different pre-incubation time, BY2 cells were treated with 100 μg mL^{-1} of E-Fol, and the activity of MAP kinases was analyzed by in-solution assay. The result shown in Figure 20B suggests that the inactivation of stimuli-induced MAP kinases by α-tocopherol is independent on antioxidant pathways, because ascorbate did not reduced the activation of MAP kinases.

Similar effect of α-tocopherol on MAP kinases activation was also observed in tobacco plants. 50 μM of α-tocopherol diluted in H_2O and only H_2O as a treated control were infiltrated into tobacco leaves and after 1, 5, and 24 hours of infiltration, activation of MAP kinases was induced by wounding. The MBP-phosphorylation activity was strongly induced by wounding in tobacco leaves infiltrated with H_2O. In contrast, wounding showed little induction of MBP-phosphorylation activity in tobacco leaves when infiltrated with α-tocopherol for 1 hour (Figure 20C). Taken together, these results strongly indicated that the accumulation of α-tocopherol can transiently inhibit stimuli-induced signal transduction pathway mediated by MAP kinases.

3. 4. 3 α-tocopherol has no direct effect on MAP kinase.

To investigate whether α-tocopherol directly inhibit the MAP kinase activity, the crude extract from BY2 cells treated with E-Fol was incubated with reaction buffer containing 50 μM of α-tocopherol or 1 μM of staurosporine, a nonspecific inhibitor of many Ser/Thr protein kinases. Staurosporine inhibited the MBP-phosphorylation activity, but α-tocopherol did not show the inhibition effect on MBP-phosphorylation activity (Figure 22). This data suggests that α-tocopherol has indirect effect on stimuli-induced signal transduction pathways.

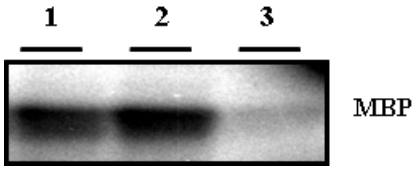


Figure 22. α-tocopherol did not inhibit the activity of MAP kinase. 5 µL of crude extract from E-Fol treated cells was incubated with reaction buffer containing 50 µM of α-tocopherol, or 10 µM of staurosprine at RT for 20 min. The MBP-phosphorylation activity was analyzed by in-solution assay. Phosphorylated MBP was visualized by autoradiography. Lane 1 presents the control without inhibitor; Lane 2, crude extract with 50 µM of α-tocopherol; Lane 3, crude extract with 10 µM of staurosprine.

The indirect effect of α-tocopherol on MBP-phosphorylation activity prompted us to investigate whether inhibitory effect of α-tocopherol is mediated by increased activity of phosphatases. The activity of phosphatases was examined by analyzing of MAP kinases inactivation using in-gel kinase assay. 20 µg of crude extract from BY2 cells treated with E-Fol was incubated at RT for 20 min with same amount of crude extract from αtE. After denaturation, samples were applied on SDS-PAGE gel contained with MBP. Figure 21B showed that the inhibitory effect of α-tocopherol was not mediated by phosphatases which can directly inactivate MAP kinases. The activated MAP kinases by E-Fol were not de-phosphorated by incubation with αtE. These data strongly suggest that the α-tocopherol regulates the inactivation of up-stream events of MAP kinases, and results in inhibiting MAP kinases activation.

3. 5 Inhibiton of MAP kinase activity by purine-based compounds

3. 5. 1 Purification of E-Fol induced MAP kinase from photoautotrophic cultured cells of tomato

To determine the inhibitor effect of purine-based compounds on the activity of plant MAP kinase, it was required to purify the active form of MAP kinase from plants as test system. It has been shown that the stimuli-induced MAP kinases which are purified from photoautotrophic cultured cells of tomato using anion-exchange chromatography have been successfully used for inhibitor study (Link *et al*., 2002a). Therefore, we induced the activation of MAP kinases from photoautotrophic cultures of tomato by treatment with 100 µg mL^{-1} of E-Fol, the elicitor preparation of the wilt-inducing fungus *F. oxysporum lycopersici*. Elicitor-induced MAP kinase was purified from the protein extract of cells treated with E-Fol for 5 min using anion-exchange chromatography. As shown in Figure 23A by in-solution assay, we were able to separate two peaks with MBP-phosphorylation activity after E-Fol treatment. To ensure that the different activity peaks eluted from the Resource Q column are due to different MAP kinases, we tested activity of MBP-phosphorylation kinase by in-gel kinase assay involved in de- and re-naturation cycles. Proteins purified by Resource Q column were subjected to electrophoresis in SDS-PAGE gel that contained the MBP. After de- and renaturation, we determined the kinase activities. Fraction Nr 17 and 18 contained high MBP-phosphorylation activities in E-Fol treated cells. In contrast, fraction Nr. 12 which contained MBP-phosphorylation activity did not contained MAP kinase (Figure 23B). Fraction Nr. 18 which contained highest MAP kinase activity was selected for further inhibitor study.

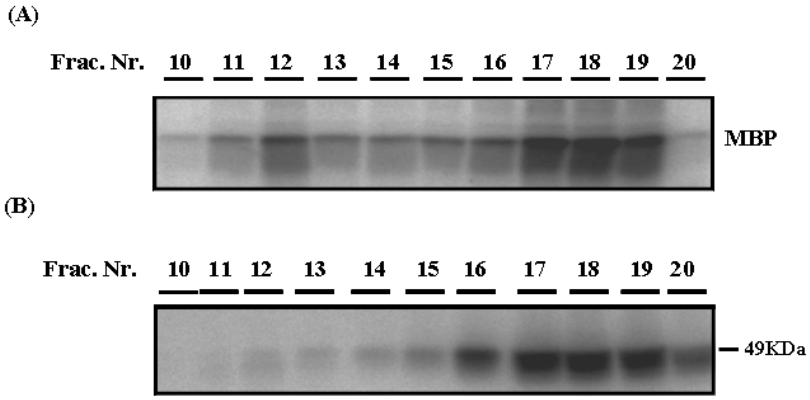


Figure 23. The major elicitor induced-MBP kinase peak eluted from the Resource Q column corresponds to the MAP kinase. Chromatography fractions 10 to 20 of E-Fol-treated cells were analyzed by in-solution assay (A) or in-gel kinase assay (B) with MBP.

3. 5. 2 The activity of plant MAP kinase is strongly inhibited by staurosporine , but not by SB203580 and ML3403

Initially we determined the inhibition effect on the activity of MAP kinase using staurosporine, SB203580, and ML3403 reported to be inhibitors of particular Ser/Thr protein kinases. Staurosporine presents two main conformational states, corresponding to a chair and a boat conformation of the tetrahydropyran ring (Furet *et al*., 1995), and is a nonspecific inhibitor of many Ser/Thr protein kinases. The pyridinyl imidazole SB203580 is an inhibitor of MAP kinases and has been extremely useful for identifying some of the physiological roles on the cell signaling pathways in mammals (Cohen, 1997). The high degree of specificity of SB203580 is indicated by its failure to affect the activities of a number of other protein kinase (Cuenda *et al*., 1995). ML3403 belongs to the second generation of diarylimidazole-type inhibitor. It has been reported to be a specific inhibitor of p38 (Kammerer *et al*., 2007). The strategy of these compounds to inhibit protein kinase is competitive with respect to ATP.

To examine the inhibition effect of staurosporine, SB203580, and ML3403, 5 μL of

MAP kinase fraction (Fraction Nr. 18) was incubated with reaction buffer containing 25 µM ATP, 1 µCi γ^{32}P-ATP, and 10 µM of staurosporine, SB203580, or ML3403. MAP kinase activity was analyzed by in-solution assay using myelin basic protein (MBP) as an artificial substrate. The results of the experiment shown in Figure 24 have been quantified; staurosporine inhibited 89.6 % of MAP kinase activity, whereas SB203580 and ML3403 inhibited 3.6 % and 9.4% of activity, respectively.

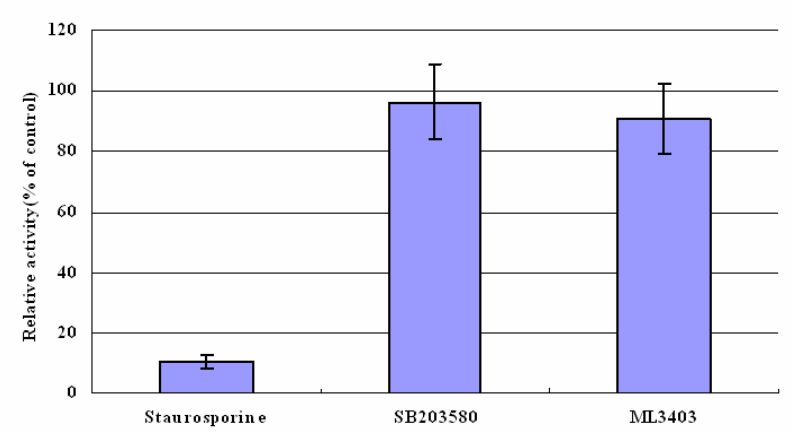


Figure 24. Effect of Staurosporine, SB203580, and ML3403 on the activity of elicitor induced MAP kinase. The activity was quantified and is depicted as percentage of the activity without addition of inhibitor (control equals 100 %). SD of three experiments is depicted.

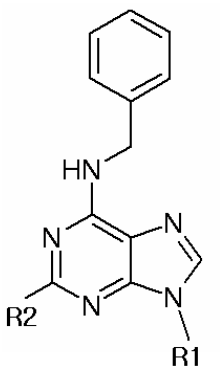
3. 5. 3 Twelve 6-(benzylamino) purine analogues display different inhibitory activity

Roscovitine, Olomucine, and Bohemine are purine analogues, but have different substitution in position C2 or N9. These compounds have been reported to have the inhibitory effect on the activity of CDKs (Vesely *et al*., 1994; Krystof *et al*., 2002; Meijer *et al*., 2003), and also shown the different potency to inhibit the activity of plant MAP kinases (Link *et al*., 2002a). This finding indicates that the minor modification of the chemical structure can have a dramatic impact on the protein kinase selectivity.

Purine analogues which are used in this study were synthesized by Strnad Lab (Institute of experimental botany ASCR in Palacky University, Czech Republic), and these compounds were tested to investigate their possibilities as plant MAP kinase inhibitors in this study for the first time.

To determine the basic relationship between their chemical structure and inhibitory effect on MAP kinase activity, initially we tested twelve 6-(bezylamino) purine compounds. The activity of MBP-phosphorylation was analyzed by in-solution assay in the presence of the inhibitors 10 μM of 6-(bezylamino) purine compounds. Figure 25 displays the different potency to inhibit the activity of plant MAP kinases. 10 μM of Bohemine, Roscovitine, A.2.1.1, A.2.1.11, A.2.1.31, B.2.1.H, and D.2.1.4 inhibited the activity of MAP kinase by 50-80 %, while other compounds had less effect on the MBP-phosphorylation activity. The (R)-(1-ethyl-2-hydroxyethyl) amino, 2-bis (2-hydroxyethyl) amino, and (2-hydroxyethyl) thio substituents at C2 caused the increased inhibitory effect, while methythio substituent in position C2 had lower effect. The different substituents at position N9 also showed different inhibitory effect on MAP kinase activity. Olomoucine and A.2.1.1 inhibited 43% and 58% of activity, respectively, whereas A.3a.1.1 did not inhibit the activity of MAP kinase.

(A)



	R1(C2)	R2 (N9)
Bohemine	(3-hydroxypropyl)amino	isopropyl
Olomoucine	(2-hydroxyethyl)amino	methyl
Roscovitine	(R)-(1-ethyl-2-hydroxyethyl)amino	isopropyl
A.2.1.1	(2-hydroxyethyl) amino	isopropyl
A.2.1.11	2-bis(2-hydroxyethyl)amino	isopropyl
A.2.1.24	[2-(2-hydroxyethyloxy)ethyl]amino	isopropyl
A.2.1.27	methylthio	isopropyl
A.2.1.31	(2-hydroxyethyl)thio	isopropyl
B.2.1.H	H	isopropyl
D.2.1.4	amino	isopropyl
A.1.1.34	N-morpholinyl	methyl
A.3a.1.1	(2-hydroxyethyl)amino	2-hydroxyethyl

(B)

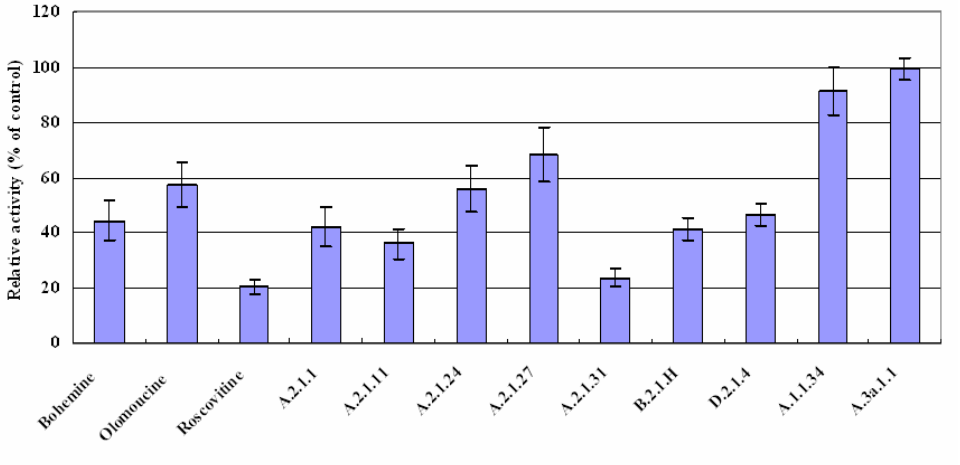
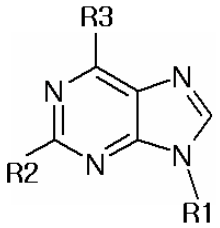


Figure 25. Effect of 6-(benzylamino) purine analogues on the activity of elicitor induced MAP kinase. (A) Chemical structure of 6-(benzylamino) purine analogues. (B) MBP-phosphorylation activity was analyzed by in-solution assay in the present of 10 µM inhibitor. The activity was quantified and is depicted as percentage of the activity without addition of inhibitor (control equals 100 %). SD of three experiments is depicted.

3. 5. 4 C2, N6, and N9-substituted purine analogues have different potency to inhibit the activity of MAP kinase.

The inhibitory effect of various purine analogues on the activity of MAP kinase was analyzed by in-solution assay in the presence of 25 μM ATP. When 10 μM of inhibitors A.2.C.6, A.2.C.Cl, A.2.2.1, A.2.P.25, A.H.8.Cl, and A.H.4.1 were used in the reaction, no inhibition in MBP-phosphorylation activity was observed, whereas A.1.2.1, A.2.3.6, and B.2.12.H inhibited the activity of MAP kinase by 40-60 % (Figure 26). The isopentenylamino, (3-hydroxybenzyl) amino, and methylamino substituents at N6 caused the increased inhibitory effect, while (4-hydroxybenzyl) amino, cyclonhexylmethylamino, and amino in position N6 did not showed inhibitory activity on MAP kinase. The presence of 10 μM A.2.C.6, 6-(benzythio) purine analogue, did not act as an inhibitor of MAP kinase whereas A.2.3.6, 6-(3-hydroxybenzy) amino purine analogue which has same substituents at position C2 and N9, inhibited 60 % of MBP-phosphorylation activity. The replacement of hydroxypropylamino group of A.2.C.6 with chloro residue (A.2.C.Cl) did not increase the inhibitory activity. Interestingly, A.1.2.1, and A.2.2.1, 6-(isopentenylamino) purine analogues, displayed significantly different effect on the activity of MAP kinase. The replacement of the N9 isopropyl group by methyl group resulted in the substantial induction of MAP kinase inhibition. 10 μM of A.1.2.1 inhibited the MBP-phosphorylation activity by 55.8 %, while A.2.2.1 inhibited 3.7 % of activity.

(A)



	R1(C2)	R3(N6)	R2(N9)
A.2.C.6	(3-hydroxypropyl)amino	benzythio	isopropyl
A.2.C.Cl	chloro	benzythio	isopropyl
A.1.2.1	(2-hydroxyethyl)amino	isopentenylamino	methyl
A.2.2.1	(2-hydroxyethyl)amino	6-(isopentenylamino)	isopropyl
A.2.3.6	(3-hydroxypropyl)amino	(3-hydroxybenzyl)amino	isopropyl
A.2.P.25	benzylamino	(4-hydroxybenzyl)amino	isopropyl
A.H.8.Cl	chloro	cyclohexylmethylamino-	H
B.2.12.H	H	methylamino	isopropyl
A.H.4.1	(2-hydroxyethyl)amino	amino	H

(B)

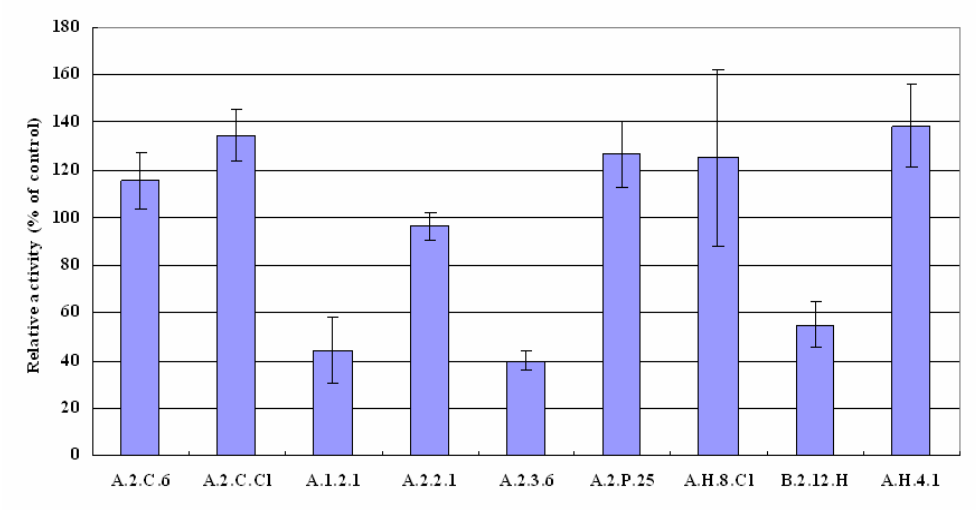


Figure 26. Effect of C2, N6, N9-trisubstituted purines on the activity of elicitor induced MAP kinase. (A) Chemical structure of C2, N6, N9-trisubstituted purines. (B) Inhibitory activity was analyzed and quantified as described in the Figure 25. SD of three experiments is depicted.

3. 6 Biotic and abiotic stress tolerance are inversely modulated by AtPDP1

Plant-derived medicines have been used for the treatment of various diseases, and suggest the possibility for development of new modern medicinal drugs (Cowan, 1999). In a previous study, the intracellular fraction of *Arabidopsis* mixotrophic cultured cells treated with SA has been shown to contain the antimicrobial activity

against *C. maltosa*. The active protein was purified from the intracellular fraction of *Arabidopsis* mixotrophic cultured cells treated with SA using gel filtration, and identified using mass spectrometry. This active protein is termed *Arabidopsis thaliana* PLAT domain protein 1 (AtPDP1) (Ali, 2007). In addition, it has been hypothesized that AtPDP1 is a possible candidate of antimicrobial peptide from the intracellular fraction of *Arabidopsis* mixotrophic cultured cells (Ali, 2007). However, further experiments are required to determine the antimicrobial activity of AtPDP1. Therefore, we have cloned and established the physiological function of AtPDP1 in plants.

3. 6. 1 Expression of *AtPDP1* in tobacco causes cell death

NCBI (National Center for Biotechnology Information) database shows that AtPDP1 shares 100 % amino acid sequence identity with lipid-associated family protein (Putative protein, Accession number: At4g39730), which contains a single PLAT domain. Therefore, gene specific primers were designed against lipid-associated family protein sequence to clone *AtPDP1*. This clone was used for our further experiment.

To investigate whether AtPDP1 is an antimicrobial peptide, we transiently transformed tobacco leaves with *AtPDP1* under the control of the CaMV35S promoter. *N. benthamiana* leaves were infiltrated with *Agrobacterium* cells carrying the *AtPDP1* construct or the transformation control (empty vector) constructs. 3 days after infiltration, protein extracts were prepared and antimicrobial activity was analyzed using paper disk diffusion method (Ezeifeka *et al*., 2004). None of all samples derived from leaves expressing *AtPDP1* showed different level of antimicrobial activity compared with treated control (data not shown). Interestingly, the leaves infiltrated with *AtPDP1* overexpression construct showed signs of tissue

collapse, and by 15 days, these infiltration zones became completely necrotic (Figure 27).

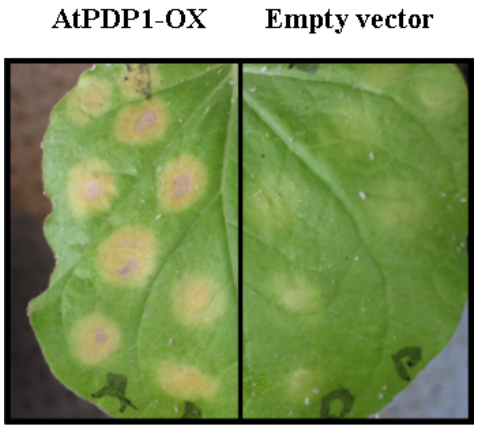


Figure 27. Transiently expressed *AtPDP1* causes the cell death in tobacco. *Agrobacterium* containing either AtPDP1-OX or empty vector (transformation control) constructs were used to transiently transform leaves of tobacco (*Nicotiana benthamiana*). Cell death was observed 15 days after agro-infiltraion.

Because H_2O_2 has emerged as an important signal in the promotion of plant cell death, we hypothesized that the expression of *AtPDP1* induces H_2O_2 accumulation in plants. To assess *in situ* the relative level of H_2O_2 accumulation induced by *AtPDP1* gene expression, the treated control and *AtPDP1* overexpressing leaves were incubated with 3,3'-diaminobenzidine solution. 2 hours after incubation, the leaves were boiled in 95% ethanol to remove chlorophyll. As shown in Figure 28A, the staining pattern revealed no detectable levels of H_2O_2 from both leaves 5 days after the infiltration. However, strong 3,3'-diaminobenzidine staining was observed in *AtPDP1* overexpressing leaves after induced cell death. Figure 28B shows the results of trypan blue dye staining, which was used to monitor cell death. HR-like cell death was characterized by localized area of dark blue staining and was clearly observed in *AtPDP1* overexpressing leaves. This staining was only observed in zone which was pressed by needleless syringe from treated control leaves.

These results suggest that overexpression of *AtPDP1* is sufficient to induce HR-like

cell death, but did not regulate the antimicrobial activity. In addition, *AtPDP1* may indirectly regulate the accumulation of H_2O_2.

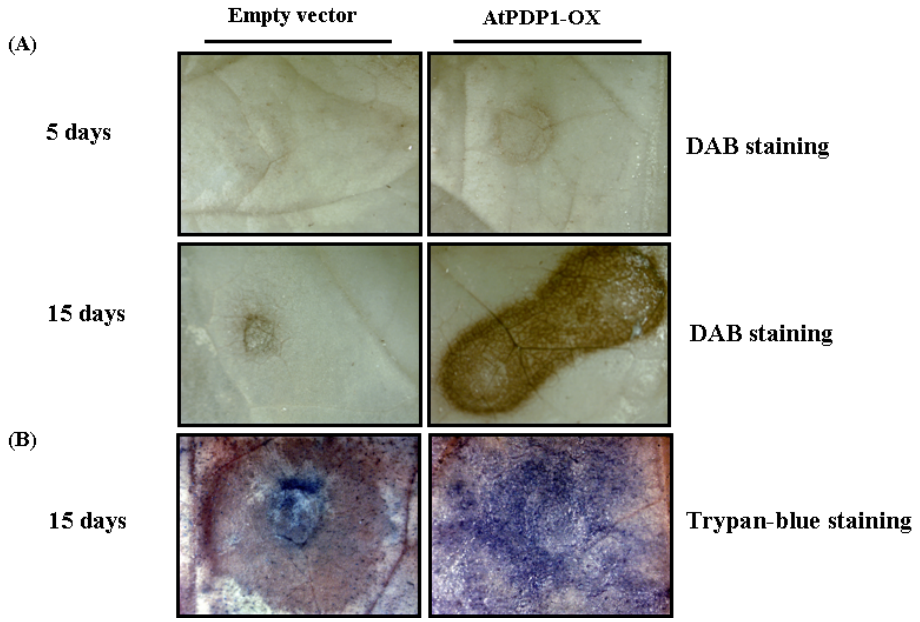


Figure 28. AtPDP1 indirectly regulated the accumulation of H_2O_2. Level of H_2O_2 and cell death was monitored using 3,3'-Diaminobenzidine (DAB) staining (A) and trypan-blue staining (B) 5 and 15 days after infiltration of AtPDP1-OX or empty vector.

3. 6. 2 *AtPDP1* gene constitutively expressed in *Arabidopsis*

To investigate the steady state transcription level of *AtPDP1* gene in different organs at different developmental stages, we isolated total RNA from *Arabidopsis* and analyzed them by RNA gel blot hybridization. As shown in Figure 29A, *AtPDP1* was constitutively expressed in different organs. In addition, *AtPDP1* transcript abundance was higher in young plants than in old plants. Low amounts of *AtPDP1* transcripts were found in the root whereas high amounts were detected in the leaves.

To determine the effect of JA and SA signaling molecules on *AtPDP1* gene expression, 1 week old *Arabidopsis* suspension cultured cells were treated with 1 mM SA or 100 μM MeJA. RNA gel hybridization showed that *AtPDP1* was down regulated

in *Arabidopsis* cultured cells treated with SA or MeJA (Figure 29B). We also observed high level of *AtPDP1* expression in non-treated cells, like seedling stage (3 week after sowing). Taken together, these data suggest that *AtPDP1* may be involved in plant growth or/and development.

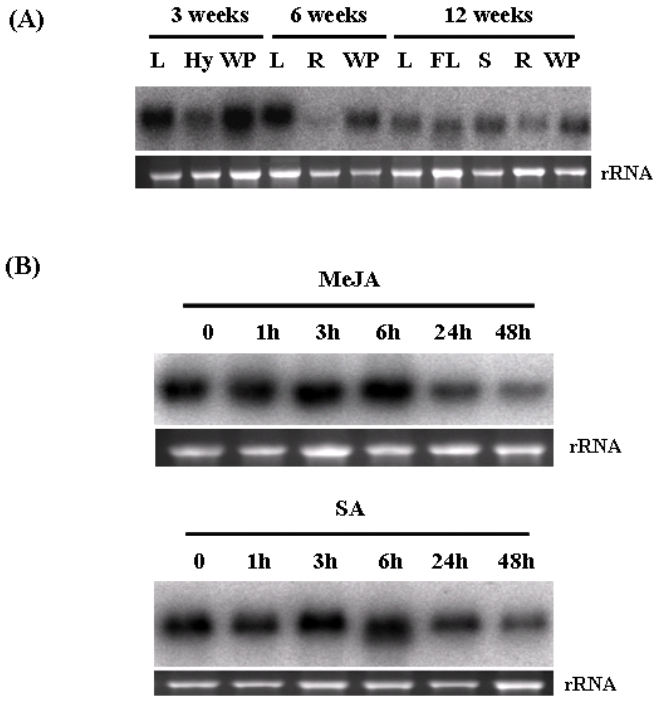


Figure 29. Expression patterns of *AtPDP1* in various tissues at different development stages and response to MeJA and SA. (A) Transcript levels of *AtPDP1* in different plant tissues at different development stages. L, Leaf; FL, Full-expanded leaf; Hy, Hypocotyls; WP, Whole plant; R, Root; S, Stem. (B) 1 week old *Arabidopsis* cultured cells were treated with 1 mM SA or 100 μM MeJA.

3. 6. 3 The expression of *AtPDP1* gene is induced by abiotic stress but not biotic stress

The member of PLAT-plant-stress subgroups was induced by different stimuli such as elicitor and dehydration. We also examined whether biotic and/or abiotic stresses are involved in the transcriptional regulation of *AtPDP1* gene. For this purpose, we inoculated *Arabidopsis leaves with P. syringae* pv. *tomato* DC3000 and RPM1 or *S.*

sclerotiorum. Total RNA was purified from samples which were taken before and 1, 3, 6, 24 and 48 h after pathogen inoculation, and subjected to gel blot hybridization (Figure 30). The biotic stresses did not induce the expression of *AtPDP1* gene. Interestingly, the expression level of *AtPDP1* gene was induced when 10 mM $MgCl_2$ was infiltrated into *Arabidopsis* leaves. We think that the increased level of *AtPDP1* gene expression may be related to abiotic stress because infiltration process itself imposes mechanical and osmotic stress.

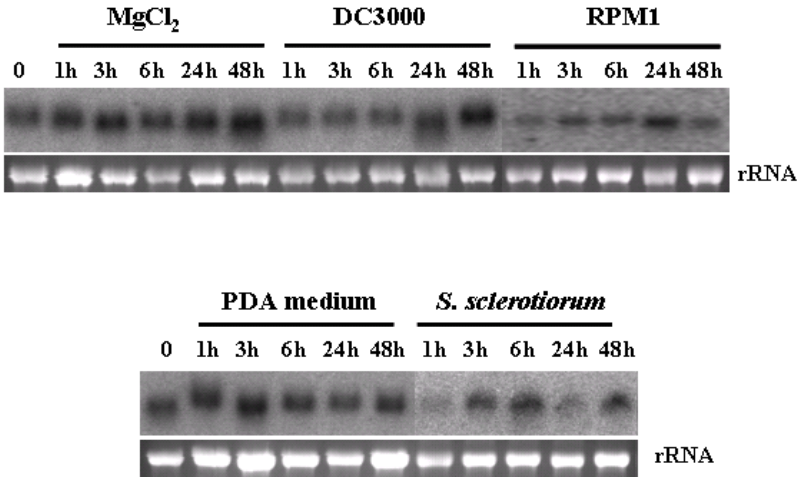


Figure 30. Expression patterns of *AtPDP1* response to biotic stresses. Leaves of *Arabidopsis* were infiltrated with 10^7 C.F.U per milliliter of *P. syringae* pv. *tomato* DC3000 or RPM1 in 10 mM $MgCl_2$. For treated control, 10 mM $MgCl_2$ was infiltrated into leaves. For *S.sclerotiorum* inoculation, an agar plug (0.4 cm in diameter) containing the advancing edge of growing mycelia was placed in the center of leaf. In parallel, leaves were mock inoculated with PDA plugs.

Significant induction of *AtPDP1* by infiltration of $MgCl_2$ prompted us to investigate the expression pattern of *AtPDP1* in response to abiotic stresses such as salinity and low temperature. RNA gel blot hybridization clearly showed that *AtPDP1* was induced by salinity and low temperature (Figure 31). In the presence of 50 mM NaCl, transcription of *AtPDP1* was increased following the time course, but this increased level of *AtPDP1* expression was similar with H_2O treatment. Expression of *AtPDP1*

gene was slowly increased within 1 h after exposure to low temperature (6°C), and peaked at 48 h. These results suggest that *AtPDP1* is likely involved in abiotic stress response rather than biotic stress response.

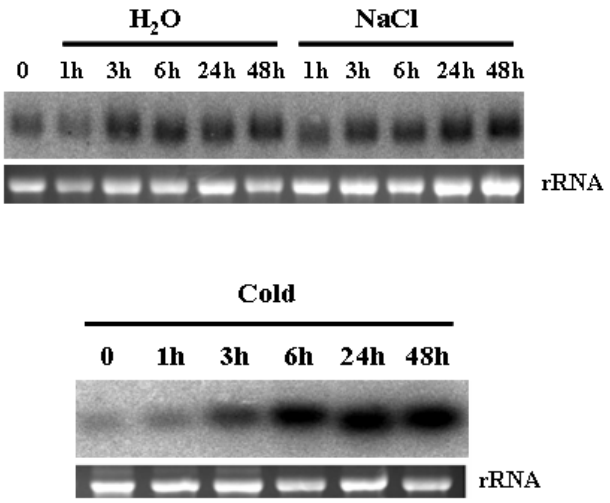


Figure 31. Expression patterns of *AtPDP1* response to abiotic stresses. Leaves of *Arabidopsis* were infiltrated with 50 mM of NaCl or H_2O. For cold stress treatment, plants were placed in a cold chamber at 6°C under white fluorescent light.

3. 6. 4 AtPDP1 localized on the endoplasmic reticulum around the nucleus

Several bioinformatics methods have been developed to predict the subcellular localization of proteins. TargetP program (Emanuelsson *et al*., 2000) predicted that AtPDP1 is a signal peptide involved in secretory pathway. To investigate subcellular localization of AtPDP1 in plants, we tried a rapid transient expression system using field garlic epidermal cells. The modified green fluorescence protein (eGFP) to *AtPDP1* is expressed by the control of 2 times cauliflower mosaic virus (CaMV) 35S promoter. The eGFP fusion constructs of full length *AtPDP1* was introduced into field garlic epidermal cells. After incubation for 24 hours, fluorescence from eGFP was examined by laser scanning microscope system (Figure 32). Field garlic cells

expressing the eGFP control showed a cytoplasmic and nuclear localization (Figure 32A). When the eGFP-AtPDP1 construct was delivered into field garlic epidermal cells, the expression was observed from the surround of nucleus (Figure 32B). This result indicates that AtPDP1 is localized in the ER surrounding this nucleus, and may be involved in the secretory pathway.

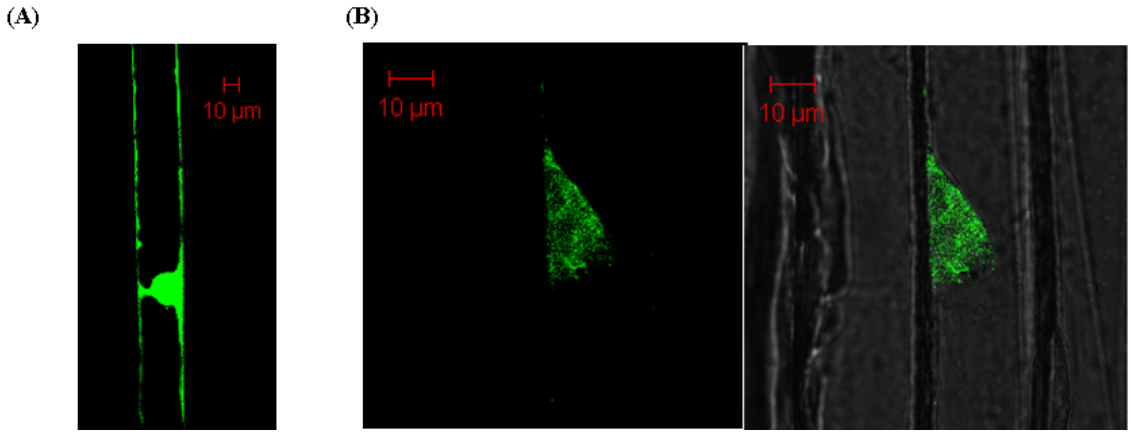


Figure 32. Localization of GFP-AtPDP1 fusion protein in Field garlic epidermal cells. Field garlic epidermal cells were either bombarded with GFP (A) or GFP-AtPDP1 chimeric protein (B). Cells were then incubated for 48h in the dark and visualized using laser scanning microscope system.

3. 6. 5 The expression of *AtPDP1* alters the growth rate

To clarify the physiological function of AtPDP1 in plants, we tried to generate the transgenic *Arabidopsis* in parallel with tobacco plants using *AtPDP1* overexpression construct, although the cell death associated with the expression of *AtPDP1* suggests that it might be difficult to generate stably transformed lines using this construct.

The cocultivation of tobacco leaf discs with the appropriate *Agrobacterium* culture and selection on kanamycin and by PCR using gene specific primers yielded a number of transgenic lines, but the expression of *AtPDP1* was detected only from three transgenic lines (Figure 33). In case of Arabidopsis transformation, so far, we

screened approximately 2500 seeds from independent *Arabidopsis* plants which were transformed by Floral dip method (Clough and Bent, 1998). However, we failed to generate the transgenic *Arabidopsis* plants overexpressing *AtPDP1*.

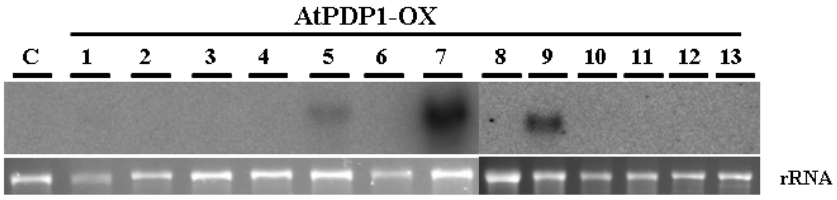


Figure 33. Screening of AtPDP1-OX lines. RNA gel blot analysis was performed using total RNA extracted from AtPDP1-OX lines (PCR-positive lines), and probed with random primer labeled cDNA fragments of *AtPDP1*. rRNA is shown as a loading control. C; empty vector transformed control lines.

These transgenic lines from tobacco plants were selected for further studies, and all of the analyses were performed on T_2 plants. The transgenic plants exhibited a significantly altered growth rate compared with transgenic control plants which were transformed using empty binary vector (Figure 34A). They were taller and had around 40% more leaves than transgenic control plants. In addition, they also showed earlier flowering. Interestingly, necrosis from unknown factors could be observed from *AtPDP1* overexpression plants in green house, whereas transgenic control plant did not show any necrosis (Figure 34B). The physiological observations suggest that *AtPDP1* overexpression resulted in improved plant growth under favorable condition. In addition, these data strongly indicated that *AtPDP1* might be related with the sensitivity to biotic factors.

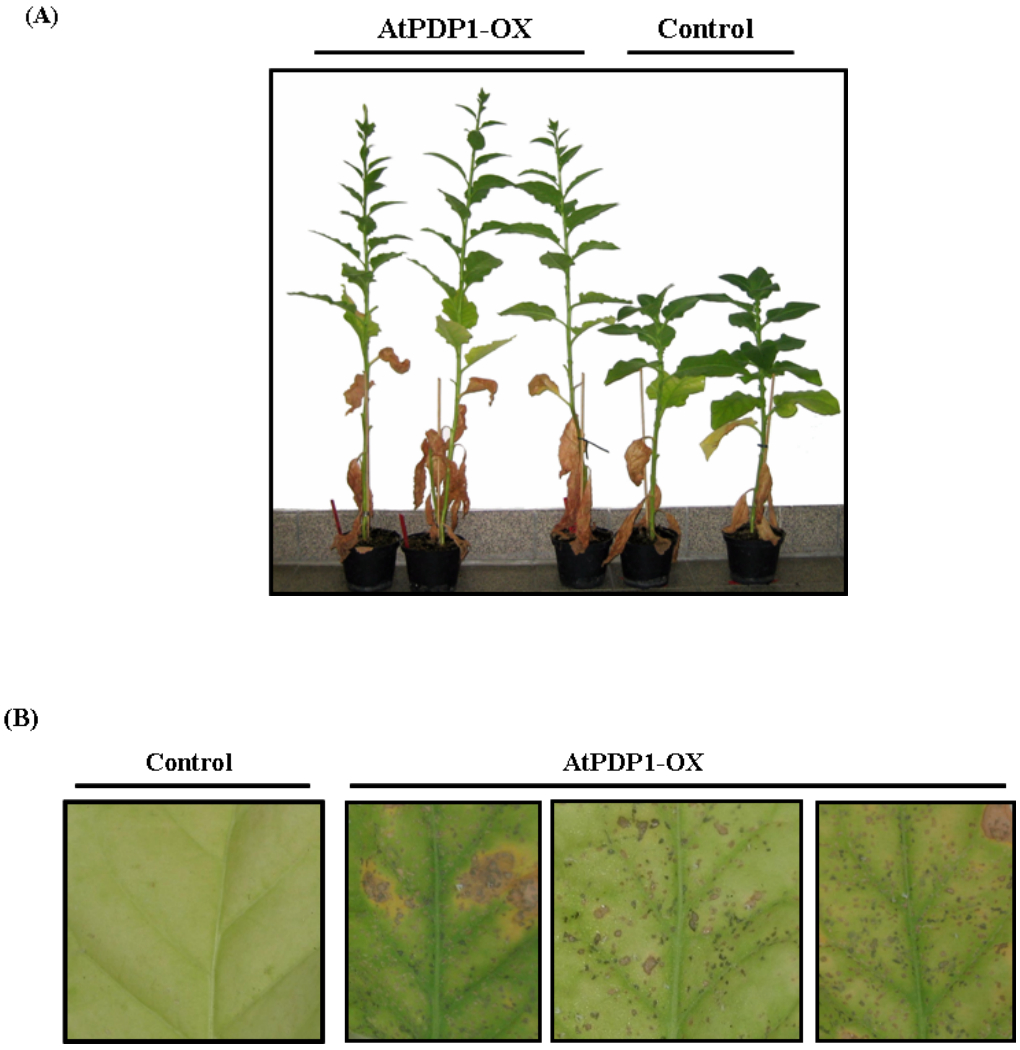


Figure 34. Overexpression of *AtPDP1* altered the growth rate of plant. (A) The height of AtPDP1-OX plants was compared with control plant. T_2 generation of both transgenic plants was photographed 3 months after growth on the soil. (B) T_2 generation of both transgenic plants was photographed 4 months after growth on the soil.

3. 6. 6 AtPDP1 enhanced the sensitivity to biotic stresses

To test the biological function of AtPDP1, we examined the effect of the expression of *AtPDP1* on host resistance against fungal and bacterial pathogens. Firstly, *P. syringae* pv. *tabaci* (virulent strain) was inoculated on plants, the growth of bacterial and development of visible symptoms were monitored. We sprayed 5 X 10^8 C.F.U. ml^{-1} *P. syringae* pv. *tabaci* in 10mM $MgCl_2$ containing 0.1% Silwet L-77 on the leaf to

induce the visible symptoms, and infiltrated 10^5 C.F.U. ml^{-1} of *P. syringae* pv*. tabaci* in 10 mM $MgCl_2$ into the leaf for bacterial growth assay. 5 days after inoculation, the visible disease symptoms were strongly developed on the inoculated *AtPDP1* overexpressing plants, and bacterial growth was significantly increased compared with transgenic control plants (Figure 35 and 36A). To monitor the spreading bacteria inside the leaf, we labeled *P. syringae* pv. *tabaci* using pPNptGreen vector, which was containing nptII-gfp fusion. The spray inoculation was not enough to detect GFP fluorescence from plants. 10^5 C.F.U. ml^{-1} of *P. syringae* pv. *tabaci* in 10mM $MgCl_2$ was infiltrated into both transgenic plants and the green fluorescence was observed using fluorescence microscopy. Figure 36B show the spreading bacteria 5 days after infiltration of GFP-labeled *P. syringae* pv. *tabaci* into transgenic control and *AtPDP1* overexpression plants. GFP fluorescence was localized on the boundary area of symptom in *AtPDP1* overexpression plants. In contrast, very week GFP expression was observed from transgenic control plant, and most fluorescence was localized inside symptom area.

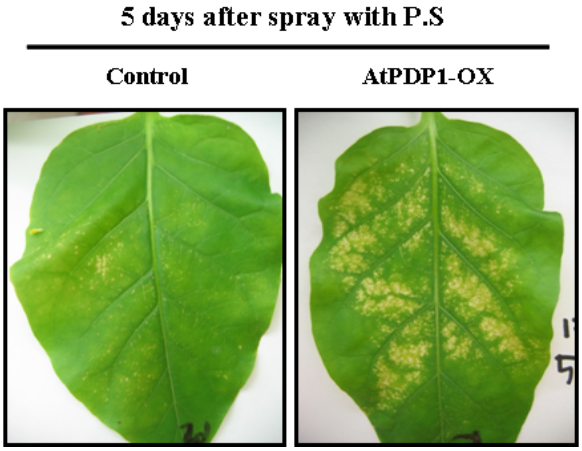


Figure 35. AtPDP1 enhanced the sensitivity to biotic stresses. 5 X 10^8 C.F.U. mL^{-1} *P. syringae* pv. *tabaci* in 10mM $MgCl_2$ containing 0.1 % Silwet L-77 were spayed on the leaf of AtPDP1-OX and control plants. Photographs show the visible disease symptoms after 5 days.

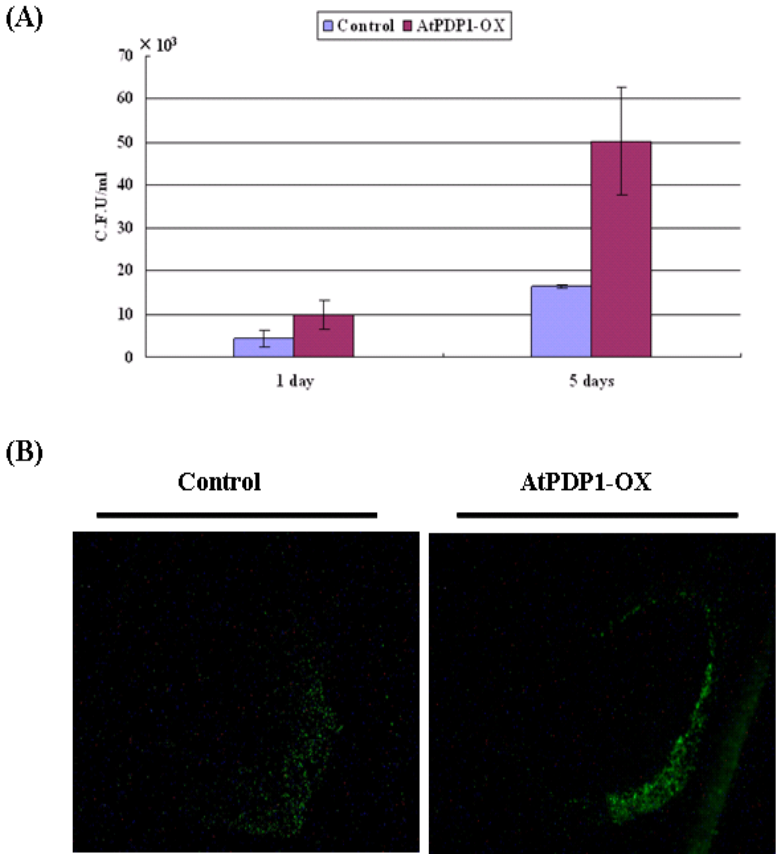


Figure 36. AtPDP1 enhanced the sensitivity to biotic stresses. (A) The number of colony forming units (C.F.U) per sample was calculated and expressed as C.F.U mL^{-1}. Experiments were done twice with similar results. (B) AtPDP1-OX and control plants were inoculated with GFP-labeled *Pseudomonas syringae* pv. *tabaci*, and GFP fluorescence was observed with UV microscope system 5 days after inoculation.

Necrotrophic pathogens have diverse pathogenesis strategies, such as production of secreting toxic substances into host cells, distinct from biotrophic pathogens. The pathogenesis strategy of *S. sclerotiorum* mediates with the secretion of oxalic acid and may induce toxic level of ROI (reactive oxygen intermediates) via the inhibition of oxidase activity (Govrin and Levine, 2000).

We assessed the regulation between *AtPDP1* expression and resistance to *S. sclerotiorum*. Figure 37 shows the symptom development from *AtPDP1* overexpression and control plants 3 days after inoculation. AtPDP1 overexpression

plants displayed the rapidly increased and strongly developed visible disease symptoms compared with transgenic control plants. Taken together, these results indicate that expression of *AtPDP1* negatively regulates the biotrophic and necrotrophic pathogens defense response.

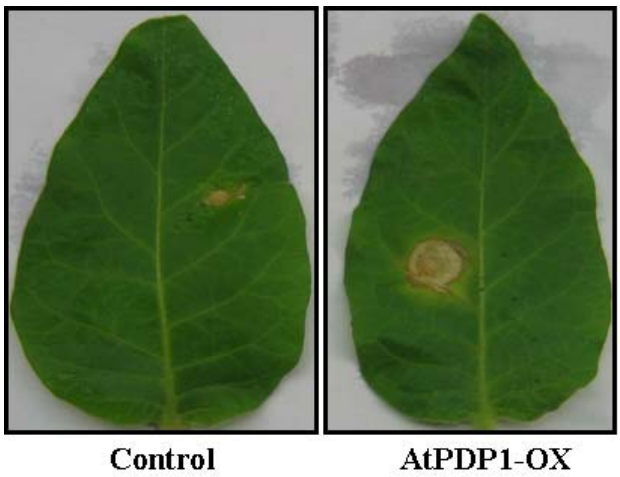


Figure 37. AtPDP1 enhanced the sensitivity to *S. sclerotiorum*. AtPDP1-OX and control plants were inoculated with *S. sclerotiorum*. Photographs show lesion after 5 days.

3. 6. 7 AtPDP1 positively regulates salt tolerance

We could not demonstrate the transcriptional regulation of *AtPDP1* against salt stress using RNA gel blot hybridization, because *AtPDP1* was also expressed by treatment of H_2O. To examine the physiological function of *AtPDP1* against high salt stress, the leaf from both plants was treated with different concentration of NaCl using exogenous feeding. 5 days after treatment with NaCl, we measured the effective photosystem II quantum yield using imaging-PAM chlorophyll fluorometer. As shown in Figure 38A, the senescence and necrosis were developed in transgenic control plants treated with 250 mM and 500 mM of NaCl. On the other hand, the similar phenotype was only observed from *AtPDP1* overepression plant when treated with

500 mM of NaCl. 5 days after treatment with 250 mM of NaCl, overexpression plants showed the delayed senescence compared with control plants and did not show necrosis. Increased concentration of NaCl was a cause of decreased level of the effective photosystem II quantum yield (Y (II)) (Figure 38B). *AtPDP1* overexpression plants contained higher level of Y (II) than transgenic control plants against salt stress. These results strongly suggest that the expression of *AtPDP1* positively regulated plant tolerance to salinity.

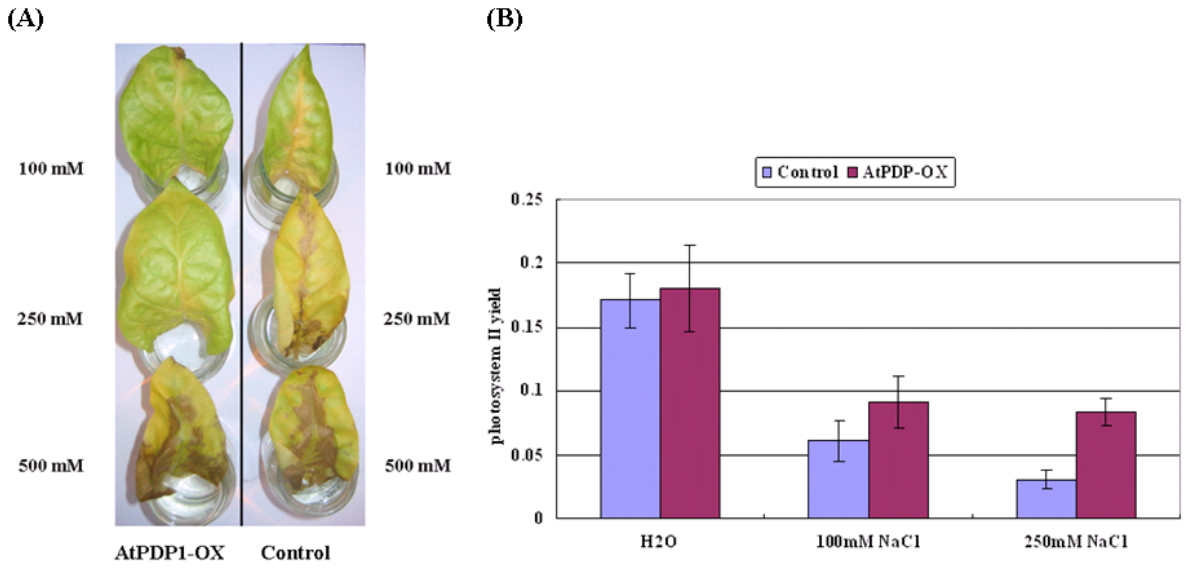


Figure 38. AtPDP1 enhanced the tolerance to salt stress. (A) The leaves from AtPDP1-OX and control plants were treated with 100, 250 and 500 mM of NaCl using exogenous feeding. The treated leaves were photographed 5 days after treatment of NaCl, and Y(II) was analyzed using an Imaging-PAM chlorophyll Fluorometer. The experiments were repeated three times with similar results.

4. Discussion

In plants, sugar production through photosynthesis is a vital process, and sucrose represents the major transport form of photosynthetically assimilated carbon. The carbohydrate metabolism and sugar signaling pathways are altered by physiological and developmental processes that have been studied in great depth over the last century. Abiotic and biotic stress-related stimuli, such as wounding, salinity, and infection by viruses, bacteria, and fungal can alter source-sink activities. Extracellular invertase, a key enzyme for hydrolyzing sucrose, is reported to be regulated by stress-related stimuli and hormones (Roitsch *et al*., 2003; Roitsch and Gonzalez 2004; Berger *et al*., 2007). This finding indicates that extracellular invertase is the central modulator of carbohydrate metabolism, stress and hormone signals, and proposes a connection between intracellular signaling and source/sink relations, although the possible link is still an open question. Thus, it will be interesting to determine whether the signal transduction pathways induced by different internal and external signals modulate and/or are modulated by source/sink relations.

Plants respond to adverse environmental conditions by activating multi-step defense responses, including production of hormones, expression of defense genes, and induction of the hypersensitive cell death. It is thought that these responses are the result of the adaptation to environmental stimuli and the perception of signal transduction in plants. Signaling through MAP kinase cascade can lead to cellular responses including cell division, differentiation as well as response to various stresses. It suggests that MAP kinase cascades act as points of convergence in stress signaling. However, negative effects of MAP kinase cascades on plant defence response have been reported. *Arabidopsis MPK4* loss-of-function mutant exhibits constitutive systemic acquired resistance (SAR) and the accumulation of salicylate

(Petersen *et al*., 2000). In addition, the expression of *PDF1.2* and *THI2.1* was blocked in *MPK4* mutant plants, whereas the *edr1* mutant (a mutation in a putative MAPKK kinase gene) has shown enhanced pathogen resistance (Frye *et al*., 2001). OsMAPK5 (Rice MAP kinase 5) positively regulates drought, salt and cold tolerance but negatively modulates the expression of *PR* gene and disease resistance (Xiong and Yang, 2003). The antagonistic regulation of MAP kinase cascades to stress responses indicates the complexity of interplay among various signaling pathways. The complexity is enhanced when activators of MAP kinase pathway, like phytohormones themselves regulate various plant responses and three-kinase module is a multigene member which involved the functional redundancy between members of the same family (Mishra *et al*., 2006).

In this study, the gain-of-function LpMPK2 and LpMPK3 were used to study the relationship between the activation of MAP kinase and the induction of Lin6 during the stress response. The identification and characterization of MAP kinases which are activated by metabolizable sugar, glucose, signal molecules, oxylipins and carbon source in source metabolism, CO_2, revealed that the activation of MAP kinase cascades plays a key role in source/sink relations. In addition, inactivation of MAP kinase in presence of α-tocopherol suggests the possible link between tocopherol signaling pathway and primary carbohydrate metabolism. They are discussed in detail below.

4. 1 The sink metabolism is a part of the MAP Kinase signaling pathway under the stress- related stimuli

In a earlier studies, it has been shown that the protein kinase inhibitor staurosporine inhibits the induction of extracellular invertase expression and the stress induced

tomato MAPK activity *in-vitro* in *Chenopodium* (Ehness *et al*., 1997) and tomato (Link *et al*., 2002a) suspension cell cultures. These findings suggested that MAP kinases may be involved in the regulation of sink metabolism by stress related stimuli, but there has been no direct evidence for this hypothesis so far. Here, we demonstrate for the first time that MAP kinase plays a critical role in the co-ordination of direct defense reactions and the regulation of sink metabolism in response to different stress related stimuli.

We selected a wound-induced protein kinase homologue LpMPK3 and a salicylic acid induced protein kinase homologue, LpMPK2 from tomato as possible candidates regulating the expression of extracellular invertase. The reason being, both SIPK and WIPK are activated by a variety of abiotic and biotic stresses indicating that they may act as a point of convergence in stress signaling. LpMPK3 showed very high sequence homology to NtWIPK and LeMPK3, while LpMPK2 showed very high sequence homology with NtSIPK and LeMPK2, both belong to subgroup A according to the classification scheme suggested for MAP kinases (MAPK group, 2002).

The time course experiments with stress-related stimuli clearly demonstrated that induction of *LpMPK3* always preceded the induction of *Lin6* transcripts (Figure 6). An increase of extracellular invertase expression in response to stress such as wounding (Roitsch *et al*., 1995), pathogens (Sturm and Chrispeels, 1990) and elicitors (Sinha *et al*., 2002), was observed in several plant species. In suspension culture of *C. rubrum*, the expression of extracellular invertase was coordinately regulated with the activation of MAP kinases by stress-related stimuli (Ehness *et al*., 1997). The same regulatory pattern was observed by non-metabolizable sugars and elicitors such as palatinose, turanose, and E-Fol in photo-autotrophic suspension cultures of *L. peruvianum* (Sinha *et al*., 2002). Nonetheless, there was no direct evidence of a relationship between induction of extracellular invertase expression and MAP kinases

activation.

Based on gain-of-function of *LpMPK3* in this study, we demonstrated that activation of *LpMPK3* led to the activation of *Lin6* promoter (Figure 9). The transiently transformed tobacco leaves harboring *Lin6* promoter::GUS gene, with *LpMPK3* showed higher activity of *Lin6* promoter under stress-related stimuli treatments. In contrast, much lower level activity of *Lin6* promoter was detected under similar conditions in control plants and transformation control plants, which in this case was an RNAi construct of LpMPK3. Interestingly, when a similar gain of function of LpMPK2 was studied, an increase in the Lin6 promoter driven GUS activity was also observed in transformation control plants. Since the *Agrobacterium* infiltration process itself imposes pathogen infection, mechanical and osmotic stress, no further increase in the *Lin6* promoter activity in transformation control plants with *LpMPK3*-RNAi while increase in GUS activity in case of *LpMPK2*-His transformation control plants (empty vector) suggests the role of LpMPK3 in the activation of Lin6 promoter. This is because *LpMPK3*-RNAi construct might have inhibited the activation of LpMPK3 thereby not leading to the activation of Lin6 promoter. Whereas, transformation with *LpMPK2*-His and empty vector might have resulted in the activation of LpMPK3 due to wounding of leaf and hence we observed higher Lin6 promoter driven GUS activity in case of LpMPK2 gain of function experiments. In alfalfa plants, rewounding does not induced the MMK4 pathway during the refractory period (Bogre *et al*., 1997). Similarly, salicylic acid application after voltage treatment dose not induced normal MAP kinase activity in photoautotrophic suspension cultures of *L. peruvianum* (Link *et al*., 2002a). These findings suggest that a second stimulation of the same pathway during the refractory period dose not lead to further activation of MAP kinase.

The present study demonstrated that the regulation of sink metabolism in general

and extracellular invertase in particular by stress-related stimuli is controlled by the activation of a MAP kinase. It is widely speculated that stress responsive MAP kinase may phosphorylate transcription factors, similar to their counterparts in animals and yeast (Tena *et al*., 2001; Zhang and Liu, 2001; Link *et al*., 2002b; Menke *et al*., 2005), but still there is no experimental evidence to support this hypothesis. Recently, ethylene responsive MAP kinases (MPK3/MPK6) from *Arabidopsis* have been shown to directly phosphorylate EIN3 transcription factor (Yoo *et al*., 2008). Interestingly a plant MAP kinase phosphorylating the plant specific enzyme, 1-aminocyclopropane-1-carboxylic acid synthase (ACS), an enzyme involved in ethylene biosynthesis has been reported (Liu and Zhang, 2004). Later on it was found that the phosphorylation of ACS is required for the stabilization of the protein (Joo *et al*., 2008). It can be speculated that LpMPK3 might phosphorylate a transcription factor which in turn is responsible for the upregulation of the extracellular invertase. The possibility of LpMPK3 directly phosphorylating Lin6 protein can be ruled out as Lin6 lacks MAP kinase phosphorylation site. Phosphorylation of tomato LpMPK3 and induction of *Lin6* promoter in tobacco leaves indicates that the upstream component and downstream targets of MAP kinases are conserved in these two genera of the Solanceae family. Elevation in *Lin6* levels and MAP kinase activation in response to stimuli have been reported separately for a number of different plant species. However, a causal relationship between these two events has so far not been established. This work shows that MAP kinase act upstream in the signaling pathways regulating both direct defence reactions and stress induced-sink metabolism in plants. Thus, MAP kinases are involved in the co-ordination of the various plant stress responses including reprogramming primary metabolism.

4. 2 AtMPK4 and AtMPK6 are important parts of the glucose and oxylipin signal transduction pathways in *Arabidopsis*

A large number of stress responsive genes have been reported to be induced by glucose, indicating the role of sugars in environmental response. Very little is known about the effect of metabolizable sugars on activation of signal transduction pathway including MAP kinase.

Photo-autotrophic cultures proved to be useful to study sugar responses (Godt and Roitsch, 1997; Sinha *et al*., 2002). Using photo-autotrophic suspension cultured cells of *C. rubrum*, it has been shown that stress-related signals and glucose signal result in the activation of MAP kinase cascades (Ehness *et al*., 1997). A similar effect of metabolizable sugars, sucrose and glucose, on the activation of MAP kinase did not occur in photo-autotrophic suspension cultured cells of tomato, whereas stress-related stimuli strongly induced the activation of MAP kinase (Sinha *et al*., 2002). Here, we further define and clarify the carbohydrate signal in MAP kinase cascade pathways by analyzing the response of photo-autotrophic suspension cultured cells of *Arabidopsis* to glucose and identification of MAP kinase using a combination of biochemical methods and immunocomplex kinase assay. The treatment of glucose resulted in the activation of 47 and 44 kDa MAP kinases. Most importantly, these MAP kinases were not activated by 50 mM of mannitol, a sugar alcohol communally used for osmotic stimuli (Figure 11). In addition, the partially purified activated MAP kinases by glucose were immunoprecipitated with specific *Arabidopsis* MPK antibodies, namely MPK3, MPK4 and MPK6 to decipher their identity. The MAP kinase activity of peak 1 and 2 were precipitated by MPK4 and/or MPK6 antibodies (Figure 15). Therefore, the separation of the MAP kinase activities by anion-exchange chromatography and the differential cross-reaction with the three MAP

kinase antibodies support that AtMPK4 and AtMPK6 are activated by glucose elicitation.

It has been shown that AtMPK6 and AtMPK3 homologs play direct or indirect roles in promoting disease resistance reactions of plants toward particular pathogens (Asai *et al*., 2002; Ekengren *et al*., 2003; Menke *et al*., 2004). On the other hand AtMPK4 kinase activity is enhanced by pathogen elicitors (Desikan *et al*., 2001) and by abiotic stresses such as cold and wounding (Ichimura *et al*., 2000). AtMPK4 is also shown to act as negative regulator of SA mediated response while it is required for the defense response of ethylene and JA (Petersen *et al*., 2000; Brodersen *et al*., 2006). In photo-autotrophic suspension cultured cells of *C. rubrum*, the treatment of staurosporine caused the inhibition of MAP kinase activity, resulted in decreased expression of extracellular invertase during the response to stress-related stimuli, whereas the glucose induced-expression of extracellular invertase was enhanced in the presence of the kinase inhibitor (Ehness *et al*., 1997). Taken together, we conclude that the metabolizable sugar independently activates the MAP kinase pathways and sink metabolism, and suggest that the differences in the induction of extracellular invertase between the carbohydrate signal and stress-related signal may be mediated by the existence of more than one signal transduction pathway.

We also demonstrated structure-specific effects of oxylipins on the activation of MAP kinase. The activation of AtMPK6 was induced by oxylipins like OPDA, PPA_1 and PGA_1, whereas no activity could be detected after treatment with MeJA, Hexenal and Hydroxy nonenal (Figure 12 and 15). It has previously been shown differences in gene induction between JA and OPDA/phytoprostanes (Muller *et al*., 2008). Similarly, the treatment with PPA_1 but not with MeJA resulted in the activation of MAP kinase, whereas the expression of Lin6 was induced by MeJA rather than PPA_1 in suspension culture of tomato (Thorma *et al*., 2003).

There are reports suggesting the involvement of MAP kinase in the induction of JA biosynthesis in plants. The overexpression of *MK*, which encodes the *Capsicum* orthologue of WIPK, elevated the level of JA in rice (Lee *et al*., 2004), and *WIPK*-, *SIPK*- and *WIPK/SIPK*-silenced plants exhibited the reduced JA production during the response to wounding (Seo *et al*., 2007). These finding, together with *Lin6* expression by JA, suggest that the production of JA is regulated with the activation of MAP kinase, and results in the induction of sink metabolism during the response to stress.

We have established AtMPK4 and/or AtMPK6 as key componets of intercellular interaction to glucose and oxylipin signal. This raises a key question as to the signaling specificity of MAP kinase during response to different stimuli. We think that the activated state of MAP kinase can determine the signaling specificity, and results in the activation of different substrates. However, further experiments are required to determine the indentification of MAP kinase substrates. Understanding the downstream signaling events of MAP kinases will ultimately help elucidate the regulatory mechanisms of intercellular signaling during response to glucose and oxylipins.

4. 3 CO_2- induced MAP kinases regulate the movement of stomata

The availability of CO_2 is a major factor for the limited photosynthesis in plants. Many examples of source/sink imbalance have been described to be associated with the level and availability of CO_2 (Farrar and Williams, 1991; Stitt, 1991; Gesch *et al*., 1998). The long-term exposure of plants to high CO_2 results in the decreased density of stomata, whereas the stomata closure is induced by the short-term exposure to high CO_2 (Roelfsema *et al*., 2002). This finding indicates that the primary resistance

to high CO_2 is mediated by the stomatal movement, and suggests the importance of stomata during plant response to CO_2.

It has been reported that the MAP kinase cascade plays a central role of the stomata movement and the guard-cell development (Shpak *et al*., 2004; Hashimoto *et al*., 2006). In *Arabidopsis*, HT1 kinase controlled the stomata movements, and its function was more pronounced in response to CO_2 than ABA and light (Hashimoto *et al*., 2006). The gain-of-function mutant of MKK4/MKK5, upstream kinases of AtMPK3 and AtMPK6 suppressed the stomata development (Wang *et al*., 2007). In addition, the NtMPK4-silenced tobacco was reported to inhibit wound-induced expression of JA-responsive genes (Gomi *et al*., 2005). Interestingly, stomata in this plant did not close in elevated atmospheric CO_2, and plasma membrane anion channels were not activated by CO_2 (Marten *et al*., 2008). Furthermore, NtMPK4 was also involved in stomatal closure response to ozone (Gomi *et al*., 2005). These findings indicated that the MAP kinase cascade is important for regulation of stomatal movement and development. Nonetheless, there was no experimental evidence of the activation of MAP kinase cascades by elevated levels of CO_2.

The present study, to the best of our knowledge is the first report about the CO_2 induced MAP kinase activation. The elevated levels of CO_2 caused the activation of 48 and 45 kDa MAP kinases in total leaf extract (Figure 16). Based on immunoprecipitation with anti-AtMPK6 and NtMPK4, we have concluded that 48 and 45 kDa MAP kinase are SIPK and NtMPK4 as key regulators of stress resistance and stomatal development (Figure 17). In *NtMPK4*-silenced tobacco plants, it has been shown the inactivation of plasma membrane anion channels during the response to CO_2, and proposed that the other MAP kinases may be activated in parallel with NtMPK4 for stomatal movement (Marten et al., 2008). It is widely speculated that CO_2 responsive MAP kinases, SIPK and NtMPK4, may activate ion channel including

K^+ channels and anion channel which regulate stomata movement, but still there is no experimental evidence whether activation of MAP kinases directly induced ion channel activation. A number of issues, however, still remain to be address such as the identification of downstream targets of SIPK and NtMPK4 during the response to CO_2 and the movement of stomata. Our study provides basic information about CO_2 induced signal transduction pathway and provides the base for future research on the regulation of stomatal movement via the activation of MAP kinase cascades.

4. 4 Non-antioxidant function of α-tocopherol on stimuli induced signal transduction pathways

Tocopherols are lipophilic antioxidants that are synthesized exclusively in photosynthetic organisms. In higher plants, α- and γ-tocopherol are predominant with their ratio being under spatial and temporal control. A number of studies suggest that the accumulation of α-tocopherol is regulated with stress tolerance, and reduction is associated with stress susceptibility (Gossett *et al*., 1994; Leipner *et al*., 1999; Munne-Bosch and Alegre, 2000). However, knockdown of γ-TMT (γ-tocopherol methyltransferase) causes an accumulation of γ-tocopherol instead of α-tocopherol, and results in increased tolerance to osmotic stress and decreased tolerance to salt stress (Abbasi *et al*., 2007). These findings indicate the complexity of interplay between tocopherol including α- and γ-tocopherol and various stress responses, and suggest that tocopherol may regulate the complex signaling pathways which are involved in plant responses to stimuli. However, our understanding of tocopherol regulated with stress-induced signaling pathways in plants is still largely unknown.

Here, we investigated the function of α-tocopherol in stimuli-induced signal transduction pathways mediated by MAP kinase. Pre-incubation with α-tocopherol

resulted in strong inhibition of elicitor-induced MAP kinase activation (Figure 20A). In addition, α-tocopherol effect on the activation of elicitor-induced MAP kinases was time dependent. Shorter or longer preincubation times like 20 min or 300 min with α-tocopherol before E-Fol stimulation yielded less inhibition of activation of MAP kinases (Figure 20A and 20C). Furthermore, our inhibition test of MAP kinase activity using α-tocopherol strongly suggest that MAP kinase is not the primary target of α-tocopherol during the stress-response in plants (Figure 22).

In mammalian cells, vitamin E including α-tocopherol has been shown to have direct and indirect effect on several enzymes involved in intercellular signaling. α-tocopherol has been shown to inhibit the effect on PKC activity and extracellular signal-regulated kinase (ERK) activation in smooth muscle cells (Clement *et al*., 1997), the activation of ERK, p38 and NF-kB in lung epithelial cells (Ekstrand-Hammarstroem *et al*., 2006). Similar inhibition effect on ERK-1 activation has been shown in human erythroleukemia cells (HEL) but not another cell lines like human monocyte tumor cell line (U937) (Breyer and Azzi, 2001). Contrary to the above effects, α-tocopherol induces the activation of ERK for increasing survival in response to H_2O_2 (Numakawa *et al*., 2006). These findings indicate that effect of α-tocopherol on signaling pathway is limited by the degree of stress, and cell types.

Several lines of evidence reported here indicate that α-tocopherol, independently its antioxidant activity, negatively regulates the stimuli-induced MAP kinase cascades pathway. First, the activity of MAP kinases was induced by TPh (α-tocopherol phosphate), whereas α-tocopherol itself did not induced MAP kinases activity (Figure 19C). TPh have been shown to perform a number of cellular functions, such as intracellular transport, cell proliferation and inflammation (Munteanu *et al*., 2004). It has been reported to protect the effect of TPh against unltraviolet B-induced damage in mouse skin (Nakayama et al., 2003) and its antioxidant activity (Rezk *et al*., 2004).

Second, stimuli-induced MAP kinase activation was reduced by α-tocopherol but not ascorbate (Figure 20B). Taken together, these evidences strongly suggest that the relationship between α-tocopherol and the activation pathway of MAP kinase cascades is not involved in antioxidant mechanism.

The inhibitory effect of α-tocopherol on stress-induced signal pathways raises a key question as to the inhibition strategy of α-tocopherol. Our data suggest that α-tocopherol directly or indirectly down-regulates the upstream signaling events like MAPK kinases and MAPKK kinases, results in reducing the activation of MAP kinases. The less activation of E-Fol induced MAP kinases was monitored from BY2 cells per-incubated with α-tocopherol using in-gel kinase assay (Figure 21A), and α-tocopherol regulates the inactivation of upstream kinases (MAPKK and MAPKKK) rather than direct inactivation of MAP kinase (Figure 21B). In mammalian cells, α-tocopherol positively regulates the activity of protein phosphatases. For example, α-tocopherol up-regulates the activity of phospho-serine/threonine phosphatase 2A (PP2A) in mouse BV-2 microglial cells (Egger *et al*., 2001), and phospho-tyrosine phosphatase (PTP) in vascular smooth muscle cells (Frank *et al*., 2000) and human neutrophils (Chan *et al*., 2001). This suggests that the effect on the inactivation of MAP kinases by α-tocopherol can be due to down-regulation of upstream signaling events via activation of protein phosphatases. Taken together, we conclude that inhibition of MAP kinases activity by α-tocopherol does not seem to be by direct interaction, but rather the result of dephosphorylation of the enzyme via activation of protein phosphatase.

Tocopherol-deficient mutant has shown the carbohydrate-accumulation phenotype during response to low-temperature, and this result suggests that tocopherol deficiency affects carbohydrate use/mobilization (Maeda *et al*., 2006). In the present study, we suggest that the activation of MAP kinase positively regulates the induction

of sink metabolism under the stress-related stimuli conditions (Figure 9). In addition, the presence of α-tocopherol suppressed the activation of stimuli-induced MAP kinase in tobacco (Figure 20A). Taken together, we hypothesize that tocopherol deficiency causes the inactivation of MAP kinase cascades, and results in the accumulation of carbohydrate during response to stress. However, further experiments are required to determine the role of MAP kinase cascades in tocopherol-deficient mutant.

Our study provides the basis for further studies and shows the effect of α-tocopherol on signal transduction pathways in plants. Even if antioxidant actions of tocopherol are quite well known, in many cases further research is required. An important issue to be addressed in the future will be to understand the complex signaling pathways which are up- and down-regulated by tocopherol, and the non-antioxidant function of tocopherol in higher plants.

4. 5 Purine-based agents are useful tools for inhibitor development

A number of protein-kinase inhibitors in small molecular weight have been used to investigate the physiological function of protein kinases, because they can be used simply and rapidly to block endogenous kinase activity. Most of all protein kinase inhibitors act by competing for the ATP binding domain of the kinase, but the basis for their specificity mediates in their interaction with residues other than those that bind ATP directly (Cohen, 1999). The structural basis for selectivity of SB compounds has been determined by comparative crystallography (Wang *et al*., 1998). In addition, these compounds have been used to identify novel physiological roles and substrates of MAP kinase (Cuenda *et al*., 1995; Ward *et al*., 2001).

Purine-based compounds have found new application as inhibitors of HSP90

(Chiosis *et al*., 2002), Src kinase (Wang *et al*., 2003), p38 MAP kinase (Wang *et al*., 1998), sulfotansferases (Armstrong *et al*., 2000), and CDKs (Havlicek *et al*., 1997). C2, N6, N9- trisubstituted purines have shown different inhibitory effect on the p34 ^{cdc2}/cyclin B kinase activity (Havlicek *et al*., 1997; de Azevedo *et al*., 1997). The different inhibitory potency of bohemine, olomoucine and roscovitine to plant MAP kinases has been reported by Link *et al*. (2002a). This finding indicated that the inhibitors can be useful for characterization of plant MAP kinase, and suggested the possibility of inhibitor development mediated by interaction between chemical structure and protein kinase structure using C2, N6, N9-trisubstituted purines.

Here, we described the relationship between chemical structure and inhibitory activity using twenty-one purine analogues which interact with the ATP-binding site of CDK. The test with 6-(benzylamino) purine suggested that the inhibitory activity is dependent on the substitution in position C2 and N9 (Figure 25). The activity of MAP kinase was strongly inhibited by roscovitine (C2: (R)-(1-ethyl-2-hydroxyethyl) amino) and A.2.1.31 (C2: (2-hydroxyethyl) thio). The same effect of substitution in position C2 from 6-(benzylamino) purine was observed by cytokinin-derived cyclin-dependent kinase inhibitors (Havlicek *et al*., 1997). This indicates that the presence of a polar side chain at position C2 may be necessary for binding to an area of the ATP binding pocket. Isopropyl and methyl substitution at position N9 of 6-(benzylamino) purine increased the inhibitory activity (Figure 25, compare olomoucine and A.3a.1.1, A.2.1.1 and A.3a.1.1). However, the increased inhibitory activity of A.1.2.1 was caused by methyl substitution rather than isopropyl substitution (Figure 26). We hypothesize that the different effects of methyl substitution on the activity of MAP kinase may be mediated by N6 substitution. However, further experiments are required to determine the role of N9 substitution in interaction between inhibitors and MAP kinases.

6-(benzythio) purine analogues did not show the inhibitory effect on the activity of MAP kinase. The hydrogen bond is important for interaction between inhibitors and ATP-binding site of protein kinase. The crystal structure of olomoucine in complex with ERK2 display the conserved hydrogen bond between Met106N and purine N7, and an additional bond from the benzylamino N to Met106O (Wang *et al*., 1998). The purine ring of 6-(benzylamino)purine analogues, olomoucine and roscovitine, bind in the same region as the purine ring of ATP, and the benzyl group contacts with outside of the ATP-binding pocket (de Azevedo *et al*., 1997). These findings indicate that benzythio S does not accept a hydrogen bond between inhibitor compound and enzyme. The hydroxybenzylamino moiety at position N6 increases CDK1/cyclin B inhibitory activity (Krystof *et al*., 2002). However, addition of hydroxyl group at the 6-(benzylamino) substituent of bohemine did not alter the inhibitory activity (Figure 25 and 26, compare bohemine and A.2.3.6).

Selectivity is a major issue of development of protein kinase inhibitors, because most of the important residues in ATP-binding site are conserved. However, our data suggest that it is possible to develop highly selective kinase inhibitors by modification of the chemical structure. This work show that C2, N6, N9-trisubstituted purines are quite useful to construct new more active MAP kinase inhibitors which regulate the structure-based selectivity.

4. 6 AtPDP1 is involved in the complex response mechanism induced by biotic and abiotic stresses

Plants have evolved a complex signaling network that involves the perception of external signals and responses to different environmental factors. Recent studies have reported the physiological function of a number of genes. However, our

understanding of the stress response in higher plants remains rather limited, considering the cross talk between different stress pathways, and unknown mechanisms by unknown proteins. Here, we have investigated the function of AtPDP1 which contain a single PLAT domain, and has an unknown physiological function in higher plants. Many of proteins contain a single PLAT domain or this domain with other functional domains such as polycystin, lipoxygenase, alpha-toxin, triacylglycerol lipase and so on (Bateman and Sandford, 1998). *AtPDP1*, which belongs to the PLAT-plant-stress subgroup, constitutively expressed in different tissues and organs at different developmental stages of *Arabidopsis* plants (Figure 29A). Interestingly, *AtPDP1* gene was highly expressed in young leaves, seedlings, and *Arabidopsis* suspension cultured cells under favorable condition (Figure 29B). Furthermore, overexpression of *AtPDP1* gene was the major cause of the accelerated plant growth rate and promoted plant aging like earlier flowering (Figure 34A). These results strongly suggest that the expression of *AtPDP1* regulates the plant growth and development.

Several studies have reported the importance of plant hormones like MeJA, ABA, ethylene, and SA in physiological processes including stress response and resistance (Seo *et al*., 1997; Anderson *et al*., 2004; van Loon *et al*., 2006). However, MeJA and SA negatively regulated the expression of *AtPDP1* gene (Figure 29B). Similarly, the introduction of *CaTin1* expression is related with ethylene but not MeJA and SA in *Capsicum annuum* (Shin *et al*., 2004). The time course experiments with biotic and abiotic stress clearly demonstrated that induction of *AtPDP1* was only related with abiotic stresses (Figure 30 and 31). An increased expression level of this subgroup gene in response to stress such as NaCl, Methyl viologen, and avirulent pathogen (Shin *et al*., 2004), was observed in several plant species. Nonetheless, there was no direct evidence of physiological function of these genes.

Multiple lines of evidence reported here suggest that AtPDP1 enhances the sensitivity to biotic stresses. First, the transiently expressed *AtPDP1* in tobacco plants caused induction of cell death, whereas empty vector control did not induce cell death. *Agrobacterium* infiltration process itself imposes pathogen infection, but is not sufficient to induce cell death in plants (Figure 27). Second, *AtPDP1* overexpression plant displayed increased susceptibility to certain virulent pathogen and necrotrophic fungus (Figure 35 and 37). Assaying the *in-planta* growth of *P. syringae* pv. *tabaci* in the transgenic plants indicated decreased basal resistance (Figure 36A). Third, necrosis from unknown factors was observed from *AtPDP1* overexpression plant, but not in transgenic control plant under favorable condition (Figure 34B).

Interestingly, *AtPDP1* positively regulates plant resistance against abiotic stresses. 5 days after treatment of salt stress, *AtPDP1* overexpression plant displayed delayed senescence and higher level of photosystem II quantum yield compared with control plants (Figure 38). Similarly, the sense and anitsense *CaTin1* transgenic plant showed enhanced tolerance to drought and salt stresses. The accumulation of ethylene in antisense *CaTin1* transgenic plants regulated the expression of *NtPR2* and *NtPR3*, and results in the resistance to various stresses (Shin *et al*., 2004). In addition, the sense *CaTin1* transgenic plants produced higher level of H_2O_2 than control plants under favorable condition. However, control plants contained 5 times higher level of H_2O_2 than sense transgenic plants when undergo HR after inoculation of TMV. As shown in Figure 28(A), the transient expression of *AtPDP1* in tobacco plants involved the accumulation of H_2O_2 during development of cell death. These findings indicate that PLAT domain protein may alter plant resistance to biotic and abiotic stresses via interfering with balance of plant hormones and H_2O_2.

Extensive study on physiological function of H_2O_2 in higher plants has demonstrated

that H_2O_2 can play a dual role in cells (Hung, *et al*., 2005). Elevation of H_2O_2 concentration in plants is a physiological response to many stimuli and result in the induction of downstream target genes. For example, *GO* (Fungal Glucose oxidase)-overexpression transgenic potato plants contain constitutively elevated level of H_2O_2 and exhibited enhanced pathogen resistance (Wu *et al*., 1997). On the other hand, transgenic tobacco plants which contained low level of antioxidant enzymes show increased level of sensitivity and general resistance against pathogen infection (Mittler *et al*., 1999). In addition, H_2O_2 is involved in signaling a variety of other hormones (Zhang *et al*., 2001; Kwak *et al*., 2006). Altered H_2O_2 levels affect ABA accumulation and ABA sensitivity (Verslues *et al*., 2007). On the other hand, ABA regulated the expression of genes related to ROS metabolism (Jiang and Zhang, 2002; Milla *et al*., 2003).

The antagonistic regulation between biotic and abiotic stress responses has also been reported in other plant species (Xiong and Yang, 2003). *OsMAPK5* (ABA-inducible mitogen activated protein kinase), activated specifically by ABA rather than by SA and JA, positively regulated plant tolerance to abiotic stresses such as drought, salinity and low temperature, but negatively modulate disease resistance and PR gene expression (Xiong and Yang, 2003). The increased level of ABA regulates antagonistic interaction between biotic and abiotic stress resistance in higher plants (Audenaert *et al*., 2002; Anderson *et al*., 2004; Thaler and Bostock *et al*., 2004; de Torres *et al*., 2007). High level of ABA enhances bacterial susceptibility and also abiotic stress resistance. In addition, ABA-deficient mutant showed enhanced defense responses against *Botrytis cinerea* (Audenaert *et al*., 2002) and *P. syringae* (virulent strain) in tomato (Thaler and Bostock *et al*., 2004) and Arabidopsis (de Torres *et al*., 2007). In addition, exogenous ABA treatment results in enhancing susceptibility of bacterial and fungal pathogens in various plant species (Mohr and

Cahill, 2003; Thaler and Bostock *et al*., 2004). Furthermore, ABA transduction involves Ca^{2+}-dependent pathway. Increased level of Ca^{2+} as a second messenger in abiotic stress and ABA response is required for the expression of some stress-induced genes in plants (Cheong *et al*., 2003).

These findings strongly suggest that the alteration of endogenous hormones and ROI regulates different response to biotic and abiotic stresses. Taken together, we hypothesize that AtPDP1 in plants may be regulated with stress response pathways via controlling level of endogenous hormones (ie., ethylene, ABA) or/and ROI like H_2O_2. However, further experiments are required to demonstrate the role of AtPDP1 in the interaction between biotic and abiotic signaling pathways mediated by altering level of endogenous hormones.

4. 7 Future perspective

A combination of molecular, biochemical, and genetic analyses has demonstrated that MAP kinase cascades play central roles in biotic and abiotic stress response, hormone response, and cell development, but the regulatory mechanisms are still unclear.

In this study, we provide the interaction of primary metabolism with stress signal transduction pathway, and suggest the basis for future research on the regulation of source/sink-relations via MAP kinase cascades. Since we report a possibility of direct or indirect regulation of Lin6 by the activation of MAP kinases, we hypothesize presence of a transcription factor or an unknown component or may be a direct link between the two. This hypothesis leads to further investigation towards understanding and characterizing the link between the activation of MAP kinase signaling cascade and induction of primary metabolism in plants.

Our finding that both AtMPK4 and AtMPK6 gets activated in response to glucose and stress treatment by immunoprecipitation with specific antibodies points towards the existence of complex cross talk in MAP kinases signaling cascade. The future course of investigation may be directed towards identifying the upstream receptors/sensors or different MAPKK and MAPKKK and downstream effectors that regulate the response. A combinatorial approach of proteomics and genomics can be taken up to answer the question. For the identification of immediate components both, upstream and downstream after the activation MAP kinase, phosphor-proteomic approach could also be implied.

Here, we suggest that α-tocopherol negatively modulates the stimuli-induced MAP kinase cascades pathway, and propose that the activation of MAP kinase cascades is a possible link between tocopherol signaling and carbohydrate accumulation phenotype. However, a causal relationship between the activation of MAP kinases and carbohydrate accumulation phenotype in tocopherol-deficient mutant has so far not been established. Therefore, an important issue to be addressed in the future will be to analyze the activation of MAP kinase cascades in tocopherol-deficient mutant. To investigate whether inactivation of stimuli induced MAP kinases is medicated by amount of α-tocopherol in BY2 cells, the measurement of uptaken α-tocopherol in BY2 cells will be required.

Our inhibitor study suggests that purine-based analogues have immense potential as chemical-biology tool to study physiological function of MAP kinases, and indicates that the structure modification of chemical compounds has influenced on their activity. The exploitation of these compounds is undoubtedly going to be of immense value in facilitating our understanding of the roles of MAP kinase in signal transduction pathways. However, this raises a key question as to the selectivity and specificity of these compounds, because purine-based inhibitors competitively interact with ATP.

Therefore, the efforts to develop the MAP kinase inhibitor need to focus on the selectivity and specificity.

The antagonistic regulation between biotic and abiotic stress responses indicates the complexity of interplay among various signaling pathways, and our understanding of plant signaling pathways involved in both stresses is still largely unknown. We show that AtPDP1 modulates enhancing sensitivity to biotic stresses and likely encodes a positive regulator of plant tolerance to abiotic stresses. This study suggests basic information about the physiological function of single PLAT domain protein under stress condition in higher plants, although we could not rule out the possible mechanisms which are modulated by PLAT domain protein. Therefore, further research will clearly be required to identify the interacting partner of AtPDP1 using yeast two hybridization, *in-vitro-binding* or other biochemical approaches. In addition, to clarify the *in-vivo* role of PLAT domain protein, analyses of gene knock-out mutant will help to elucidate the physiological function and molecular mechanisms.

In this study, we provide the relationship between stress signal transduction pathway and the primary metabolism, and suggest the basis for future research on the regulation of source/sink-relations via MAP kinase cascades. In addition, the characterization of AtPDP1 suggests the complex defense response in plants. From this point of view studying signal transduction mediated by MAP kinase cascades and plant defense mechanism might help us in better understanding of plant physiology.

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Appendix

1. LpMPK3 sequence

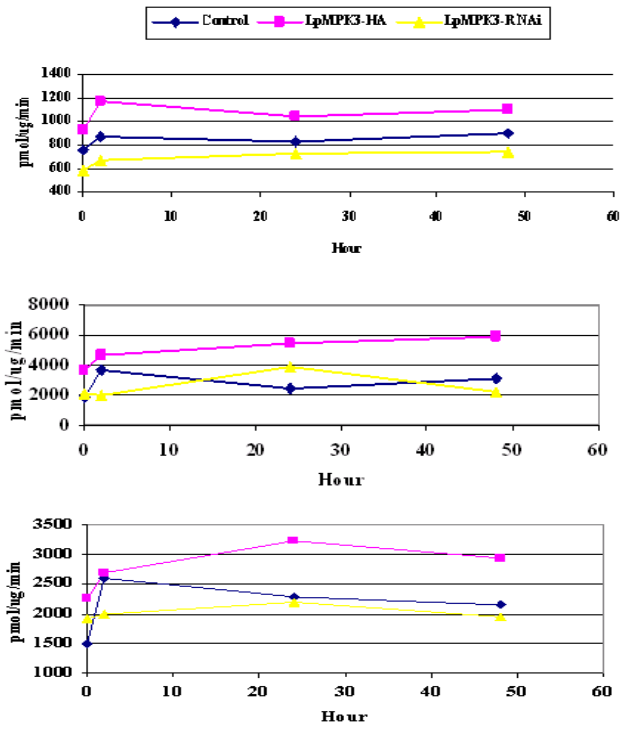
DEFINITION	Solanum peruvianum mitogen-activated protein kinase 3 (MPK3) mRNA,complete cds.
ACCESSION	EU178755
SOURCE	Solanum peruvianum (Lycopersicon peruvianum)
AUTHORS	Hoffmann,A., Hyun,T., Sinha,A.K. and Roitsch,T.
TITLE	Mitogen-Activated Protein Kinase 3 (LpMPK3) from Lycopersicon peruvianum
JOURNAL	Submitted (26-SEP-2007) Pharmaceutical Biology, Julius von-Sachs Institute, Julius-von-Sachs Platz-2, Wuerzburg, Bavaria 97082, Germany
Translation	MVDANMGAAQFPDFPKIVTHAGQYVQYDIFGNLFEITNKYQPPIMPIGRGAYGIVCSVFNAELNEMVAVKKIANAF DNYMDAKRTLREIKLLRHLDHENVIGLRDVIPPPLRREFSDVYIATELMDTDLHQIIRSNQGLSEDHCQYFMYQLL RGLKYIHSAHVIHRDLKPSNLLLNANCDLKICDFGLARPNVENENMTEYVVTRWYRAPELLLNSSDYTAAIDVWSV GCIFMELMNRKPLFAGKDHVHQIRLLTELLGTPTESDLSFLRNEDAKRYVRQLPQHPRQQLATVFPHVNPLAIDLV DKMLTLDPTRRITVEEALAHPYLAKLHDAADEPVCPIPFSFDFEQQGIGEEQIKYMIYQEALALNPEYA
ORIGIN	1 atggttgatg ctaatatggg tgctgctcaa tttcctgatt ttcctaaaat tgtcactcat 61 gctggacaat atgttcagta tgacattttt ggtaatcttt ttgagattac taacaagtat 121 caacctccta ttatgcctat tggacgtggc gcttatggaa tcgtctgctc tgtgtttaat 181 gcggagctga atgagatggt tgcagttaag aaaatcgcca atgcttttga taattacatg 241 gatgctaaga ggacgctccg tgaaattaag cttcttcgcc atttagacca tgaaaacgtc 301 attggtttaa gagatgtgat tcctccgccc ttacgaaggg agttttctga tgtttacatt 361 gctactgaac tcatggatac tgatcttcac caaataatta gatcaaacca aggtttatca 421 gaggatcatt gccagtactt catgtatcag cttctccgtg ggctaaagta catacattcc 481 gcgcatgtta ttcatagaga tctcaaacca agtaacctct tgctaaatgc aaattgtgat 541 cttaagatat gtgattttgg tcttgcaagg ccaaacgtag agaacgagaa tatgacagaa 601 tatgtagtaa ccagatggta cagggcaccg gagcttttgt tgaactcttc agattacact 661 gctgccatag atgtttggtc tgtgggttgc atcttcatgg agcttatgaa tagaaaacct 721 ttgtttgctg gaaaagatca tgtacatcaa atacgcttgc taactgagct tcttggcact 781 cctacagaat ctgatcttag cttcctccgt aatgaagatg caaaaagata cgtcaggcaa 841 ctcccacaac atccacgcca gcagttagcg acagtgttcc ctcatgtgaa tccattagcc 901 attgatcttg tagataagat gttgacgctc gaccctacta gaagaataac agttgaggaa 961 gcattagctc atccctacct cgcaaagctc catgatgcag ctgatgaacc agtctgcccc 1021 atcccgttct ctttcgactt tgagcaacaa gggataggag aagagcagat taaatacatg 1081 atttatcaag aagctttggc gttgaatcct gaatatgctt aa

2. LpMPK2 sequence

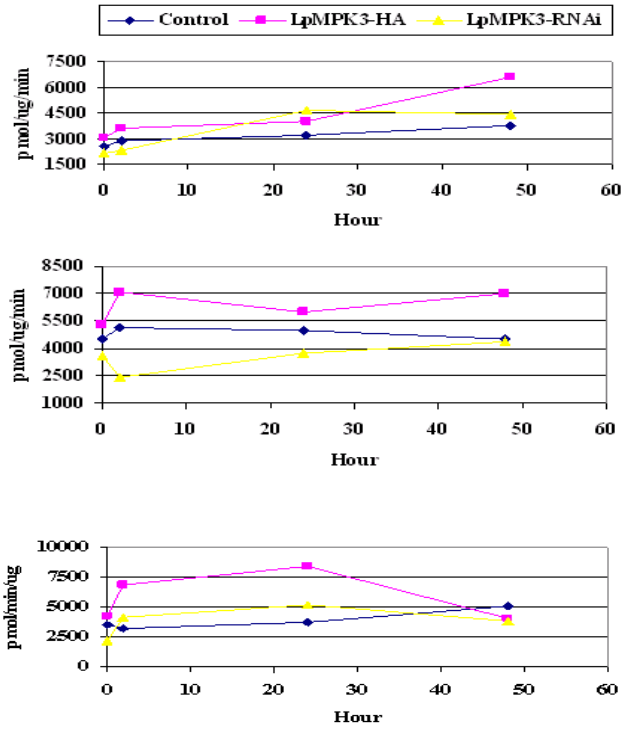
DEFINITION	DEFINITION Solanum peruvianum mitogen activated protein kinase 2 (MPK2) mRNA,complete cds.
ACCESSION	EU887292
SOURCE	Solanum peruvianum (Lycopersicon peruvianum)
AUTHORS	Hyun,T., Sinha,A.K. and Roitsch,T.
TITLE	Mitogen-Activated Protein Kinase 2 (LpMPK3) from Lycopersicon peruvianum
JOURNAL	Submitted (15-JUL-2008) Pharmaceutical Biology, Julius von-Sachs Institute, Julius-von-Sachs Platz-2, Wuerzburg, Bavaria 97082, Germany
Translation	MDGSAPQTDTVMSDAAAGQQPAMPPLPMAGMENIPATLSHGGRFIQYNIFGNIFEVTAKYKPPIMPIGKGAYGIVC SALNSETNEHVAIKKIANAFDNKIDAKRTLREIKLLRHMDHENIVAIRDIIPPPQREAFNDVYIAYELMDTDLHQI IRSNQGLSEEHCQYFLYQILRGLKYIHSANVLHRDLKPSNLLLNANCDLKICDFGLARVTSETDFMTEYVVTRWYR PPELLLNSSDYTAAIDVWSVGCISMELMDRKPLFPGRDHVHQLRLLMELIGTPSEAEMEFLNENAKRYIRQLPLYR RQSFVEKFPHVNPAAIDLVEKMLTFDPRRRLTVEDALAHPYLTSLHDISDEPVCMTPFSFDFEQHALTEEQMKELI YREGLAFNPEYQHM
ORIGIN	1 atggatggtt cagctccgca aacggatacg gtgatgtcgg atgcggcggc gggacagcaa 61 cctgctatgc cgccgctgcc gatggccgga atggagaata ttcctgcaac gttgagtcac 121 ggtggccggt ttattcaata caatattttt ggtaatatat ttgaagttac tgcgaagtat 181 aagcctccaa ttatgccaat cggtaaagga gcttatggta ttgtttgttc tgctttgaat 241 tcggagacga atgaacatgt tgcaataaag aaaattgcaa atgcttttga taacaaaatt 301 gatgccaaga gaactttgcg tgagatcaag cttcttcgac atatggatca tgaaaatatt 361 gttgcgatta gagatataat tccaccacct caacgagaag catttaatga tgtctatatt 421 gcatatgagc ttatggacac tgatctccat caaattattc gctctaatca gggattatca 481 gaagaacatt gccagtattt cttgtatcag atcctccgtg gattgaaata catacattct 541 gcaaatgtgt tgcataggga cttgaagcct agcaatctcc tcttgaatgc caattgtgat 601 ttaaagatat gtgattttgg gctagctcgt gtcacttctg aaactgactt tatgaccgaa 661 tatgttgtga caagatggta ccgacctcca gagctgttgt taaattcttc tgactatact 721 gcagctattg atgtttggtc agttggttgc atttccatgg aactgatgga cagaaagcct 781 ctgtttcctg gtagagatca tgtacaccag ctacgtctgc ttatggagtt gattggcacc 841 ccgtctgagg ctgaaatgga gtttttgaat gagaatgcaa agcggtacat cagacaactt 901 cctctttacc gtcgacaatc atttgtcgaa aaatttccac atgtaaaccc tgctgctatt 961 gatcttgttg agaagatgtt gacatttgat cccagaagga gacttactgt tgaagacgcg 1021 cttgctcatc cttacctaac atcgcttcat gatattagtg acgagcccgt ttgcatgact 1081 ccttttagct tcgattttga acagcatgcc cttaccgagg aacaaatgaa agagctgatt 1141 tacagggagg gtcttgcatt taatcccgaa taccagcaca tgtga

3. LpMPK3 leads to expression of Lin 6 promoter after treatment of stimuli

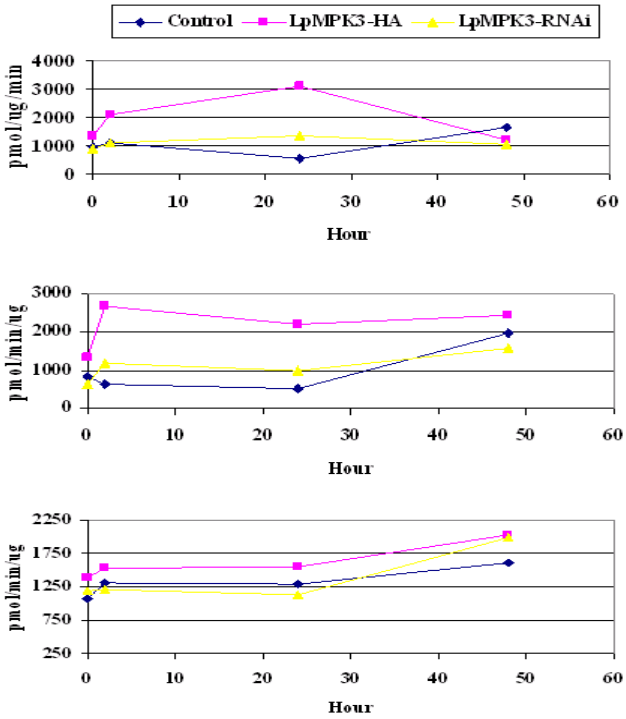
3. 1 Wounding



3. 2 E-FOI



3. 3 KCl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LpMPK3-RNAi
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4000
3000
2000
1000
0
0 10 20 30 40 50 60
Hour
pmol/min/ug
3000
2000
1000
0
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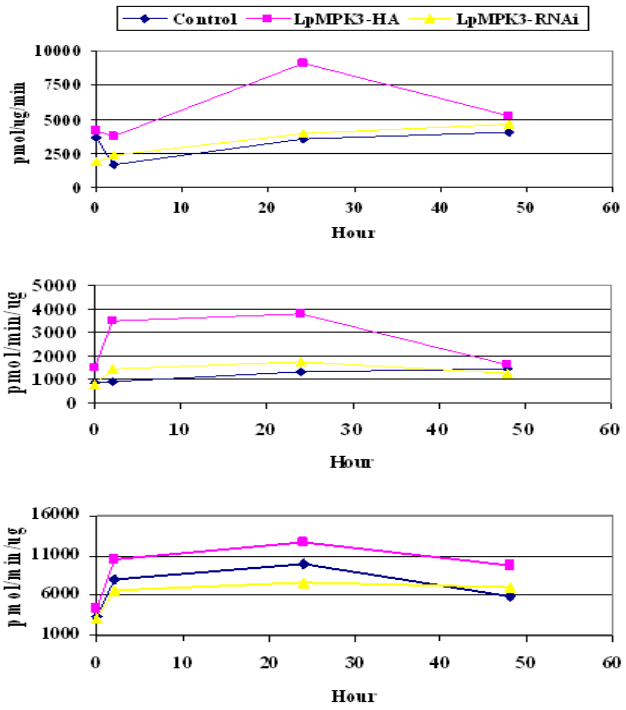
Hour
pmol/min/ug
2250
1750
1250
750
250
0 10 20 30 40 50 60
Hour

3. 4 PGA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LpMPK3-RNAi
pmol/ug/min
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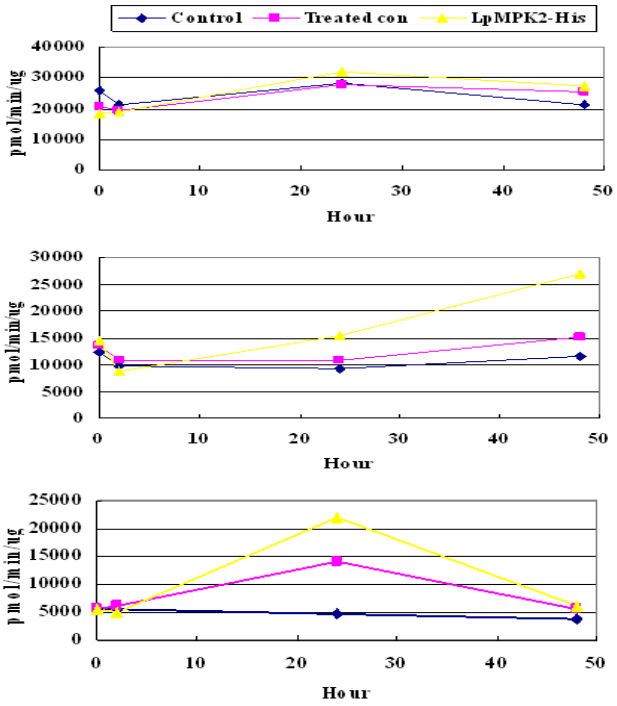
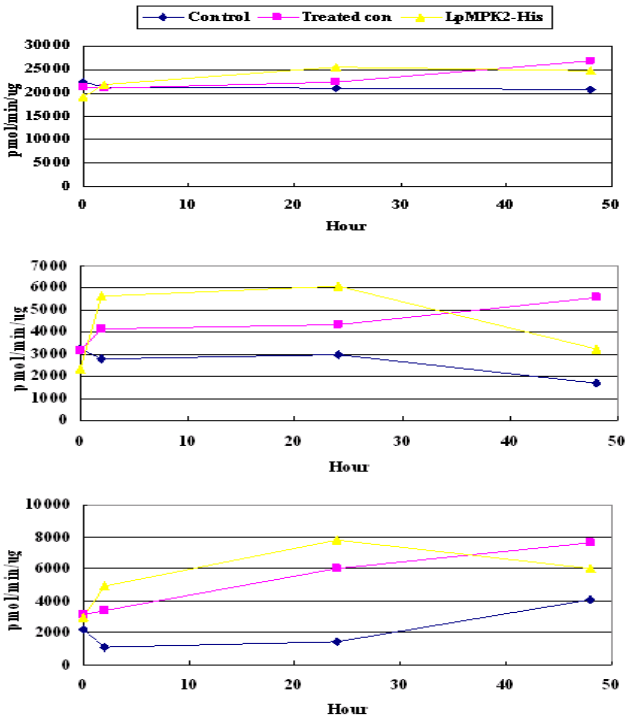
7500
5000
2500
0
0 10 20 30 40 50 60
Hour
pmol/min/ug
5000
4000
3000
2000
1000
0
0 10 20 30 40 50 60
Hour
pmol/min/ug
16000
11000
6000
1000
0 10 20 30 40 50 60
Hour

4. LpMPK2 leads to expression of Lin 6 promoter after treatment of stimuli

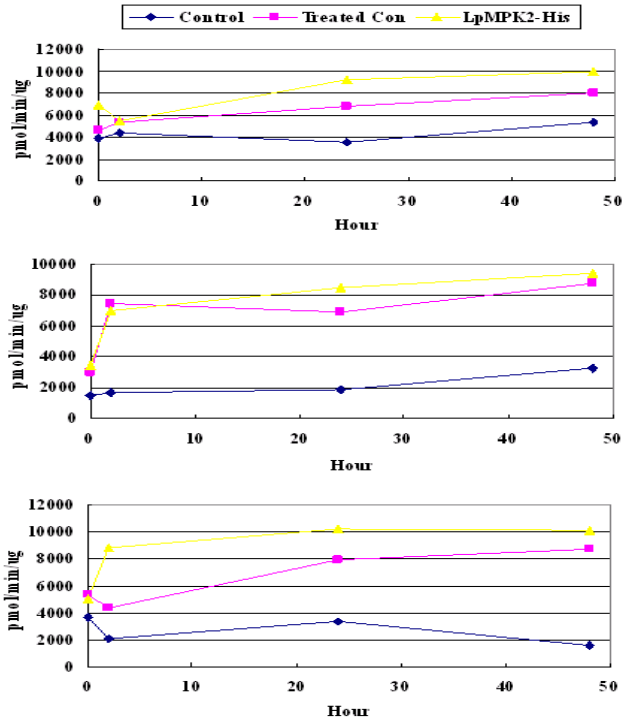
4. 1 Wounding



4. 2 E-Fol



4. 3 KCl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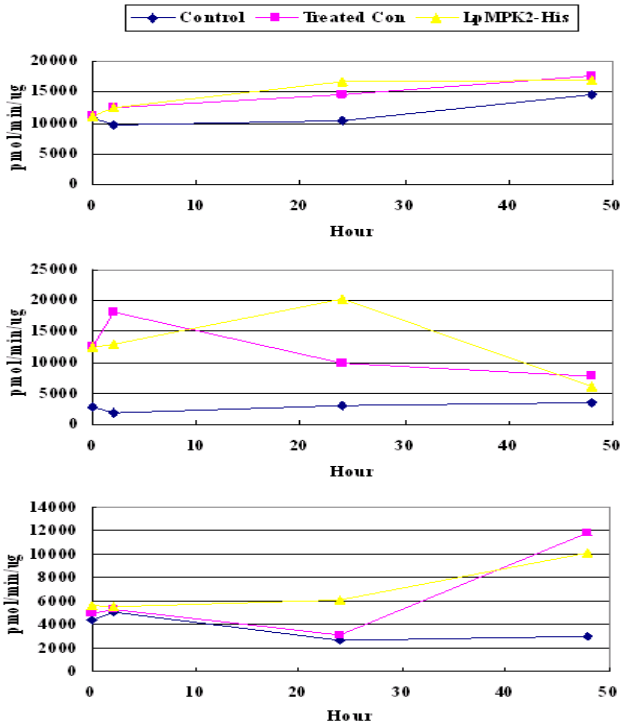
Treated Con
LpMPK2-His
pmol/min/ug
Hour

4. 4 PGA


Control
Treated Con
LpMPK2-His
pmol/min/ug
Hour

Publications

Marten, H., Hyun, T. K., Gomi, K., Seo, S., Hedrich, R., and Roelfsema, M. R. G. (2008). Silencing of NtMPK4 impairs CO_2-induced stomatal closure, activation of anion channels and cytosolic Ca^{2+} signals in Nicotiana tabacum guard cells. Plant J. 55: 698-708. *

Hyun, T. K., Hoffmann, A., Sinha, A. K., and Roitsch, T. Tomato mitogen activated protein kinases regulate the expression of extracellular invertase Lin6 in response to stress related stimuli. (submitted to Functional Plant Biology)

Hyun, T. K., Kumar, K., Rao, K. P., Franke, M., Sinha, A. K., and Roitsch, T. Non-antioxidant function of α-tocopherol in plants: α-tocopherol inhibits stress-induced MAPK activation. (submitted to FEBS lett)

Publications marked with an asterisk are not part of this thesis

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