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In-silico screening of WRKY transcription factors as possible substrates of mitogen activated protein kinase 3 in *Solanum lycopersicum*

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Abstract

Activation of mitogen-activated protein kinase (MAPK) is a common reaction of plants in defense-related signal transduction pathways. However, little is known about MAPK signaling pathways during stress response due to limited knowledge about plant MAPK substrates. In this study we present a list of WRKY transcription factors as possible substrates of tomato LeMPK3. Immuno-complex kinase activity assay and in-gel kinase assay showed that a set of three different stimuli (wounding, the endogenous plant-derived elicitor PGA, and salt stress) results in the fast and transient activation of both LeMPK2 and LeMPK3 within 5 minutes. Based on gene expression patterns and co-expression analysis obtained by microarray experiments, we have identified three WRKY proteins as substrates of LeMPK3 during response to different stimuli. The predicted LeMPK3-WRKYs network will help to understand the function of MAPK in stress-related stimuli signaling pathways.

Keywords: MAPK; LeMPK3; WRKY; Co-expression; *In-silico* analysis.
Abbreviations: MAPK- Mitogen Activated Protein Kinase; MBP- Myelin Basic Protein; PGA- Polygalacturonic Acid.

Introduction

In higher plants, a large variety of protein kinases are involved in controlling intracellular responses to extracellular information. One of the largest categories of protein kinases is mitogen-activated protein kinase (MAPK) cascade, which is a major downstream component of receptors/sensors. MAPK is the last component of the MAPK cascade (MAPKKK-MAPKK-MAPK) and plays crucial roles in signal transduction pathways that lead to the activation of cellular responses. Since MAPK genes were first reported in peas in 1993 (Stafstrom et al., 1993), a number of MAPKs have been characterized in plants such as Arabidopsis, rice, and tobacco. As far as we know, plant MAPKs contain TEY or TDY motif located in the activation loop between kinase subdomains VII and VIII, and they regulate not only biotic and abiotic stress responses, but also plant growth and development (Tena et al., 2001; Lampard et al., 2009; Pitzschke et al., 2009). A total of 20 MAPKs and their immediate upstream regulators, 10 MAPKKs, and more than 60 MAPKKKs have been identified in the Arabidopsis genome (MAPK group, 2001). The best-characterized Arabidopsis MAPKs are AtMPK3, AtMPK4, and AtMPK6, all of which are activated by stress-related stimuli, including abiotic and biotic stress. Although the accumulated experimental evidence suggests an important physiological role for plant MAPK cascades (Asai et al., 2002; Wang et al., 2007; Pitzschke et al., 2009; Kishi-kaboshi et al., 2010), little is known about each combination of plant MAPK cascades and the specific roles that each MAPK cascade gene plays in particular plant signal transduction pathways. In addition, the understanding of MAPK-mediated responses to stress is limited by the current knowledge of plant MAPK substrates. Gene expression patterns obtained by microarray experiments have recently been used as tool to predict the gene-to-gene functional relationships (Obayashi and Kinoshita, 2010), indicating that co-expression approaches might be applied to the prediction of biological targets, including subunits of protein complexes and enzymes in metabolic pathways. Thus, we have analyzed tomato gene expression profiles to identify the downstream of stress-induced MAPK during response to stimuli.

Results and discussion

To analyze the activation pattern of tomato MAPKs, we infiltrated tomato leaves various times with 50 mM NaCl or 100 μg μL^{-1} polygalacturonic acid (PGA). PGA is a host-derived elicitor that is a degradation product of plant cell walls; the treatment of PGA resulted in induction of MAPK activation in tomato suspension culture cells (Link et al., 2002). Protein extracts were prepared and kinase activity was determined by immuno-complex kinase activity assay with phosphotyrosine-specific mono-cloned antibody 4G10. As shown in Fig. 1A, NaCl and PGA induced activation of tomato MAPKs within 5 min, and maximum activation of MAPKs was observed 10 min after treatment with these stimuli. After wounding, a transient and rapid activation of tomato MAPKs could be observed. To further characterize the activation of tomato MAPKs activated by treatment with NaCl, PGA, and wounding, in-gel kinase assays with MBP were performed, which revealed the activation of two MAPKs with different molecular masses (Fig.

Table 1. The list of probe sets for *WRKY*, *LeMPK2* and *LeMPK3*.

Protein	Probe	Expect	Identities	Target length (bp)
LeMPK2	Les.4302.1.S1_at	0.0	99.4%	1573
LeMPK3	Les.4316.1.S1_at	0.0	99.2%	1248
PTLe00810	Les.546.1.A1_at	0.0	99.7%	351
PTLe00817	Les.3964.1.S1_at	0.0	100.0%	1253
PTLe00820	LesAffx.735.1.S1_at	0.0	99.8%	466
PTLe00822	LesAffx.36712.1.S1_at	0.0	99.0%	985
PTLe00831	Les.5444.1.S1_at	0.0	99.3%	1154
PTLe00834	LesAffx.21820.1.S1_at	0.0	98.8%	980
PTLe00838	LesAffx.43341.1.S1_at	0.0	99.2%	711
PTLe00843	Les.3969.1.S1_at	0.0	96.5%	606
PTLe00844	LesAffx.43341.1.S1_at	0.0	98.3%	711
PTLe00847	LesAffx.51257.1.S1_at	0.0	100.0%	706
PTLe00854	Les.2667.2.S1_at	0.0	99.6%	1419
PTLe00856	LesAffx.837.1.S1_at	0.0	100%	935
PTLe00859	LesAffx.4793.1.S1_at	0.0	97.1%	841
PTLe00860	LesAffx.9910.1.S1_at	0.0	98.8%	1478

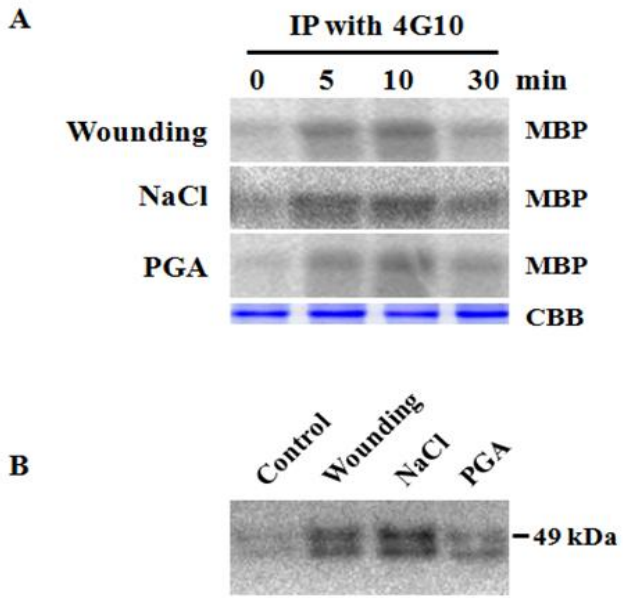

Fig 1. Rapid activation of mitogen-activated protein kinase due to stress treatment. (A) The protein kinase activity was analyzed by immuno-complex kinase activity assay with 4G10 antibody . The lower panel shows Coomassie Brilliant Blue (CBB) staining for equal loading of the proteins. (B) MAPK activity was analyzed by in-gel kinase assay using MBP as substrate. Crude extract of untreated leaves (Control) or leaves treated with wounding, NaCl, or PGA were analyzed by in-gel kinase assay. All experiments were repeated three times with similar results

1B). Sixteen MAPK family members have been identified in tomato genome, of which LeMPK1, LeMPK2, and LeMPK3 were shown to be involved in stress-induced signal transduction pathways (Kandoth et al., 2007; Stulemeijer et al., 2007). Similarly, it has been shown that LpMPK2 and LpMPK3 are activated by wounding, KCl, and elicitors in tobacco (Hyun et al., 2009). These findings are in agreement with our finding that treatments with NaCl, PGA, and wounding lead to activation of at least two different MAPKs in tomato (Fig. 1B). Based on the activation pattern and molecular mass, we propose that NaCl-, PGA-, and wounding-induced tomato MAPKs are LeMPK2 (homologous to LpMPK2) and LeMPK3 (homologous to LpMPK3). WRKY proteins are a large group of transcription factors that are classified as zinc-finger proteins. It has been shown that WRKY proteins regulate the alteration of gene expression in response to pathogens, viruses, wounding, and drought stress (Ross et al., 2007; Kumar et al., 2009; Kamal et al., 2010). Within these networks, several WRKY proteins are suggested as possible substrates of MAPKs in Arabidopsis, tobacco, and rice (Ross et al., 2007). We downloaded 52 *WRKY* mRNAs and protein sequences from Plant Transcription Factor Database to identify the downstream of stress-induced tomato MAPK during response to stimuli (Fig. 2). The WRKY family contains 74 genes and 102 genes in Arabidopsis and *indica* rice (Eulgem et al., 2000; Ross et al., 2007), respectively, that are placed into four subgroups based on the number of WRKY domains and the features of their zinc-finger-like motif (Ross et al., 2007). Tomato WRKY proteins were also placed into four subgroups. For example, PTLe00820, PTLe00829, PTLe00837, PTLe00854, and PTLe00857 contain two WRKY domains and zinc-finger-like motifs (C–X4–5–C–X22–23–H–X1–H) and are defined as Group I. PTLe00812, PTLe00822, PTLe00838, PTLe00839, PTLe00845, PTLe00856, and PTLe00859 have one WRKY domain and a C2–HC motif (C–X7–C–X23–H–X1–C) defined as Group III. Although Group IV WRKY proteins contain the

Table 2. WRKY transcription factors that are closely co-expressed with LeMPK3.

WRKY	r value	p value
PTLe00854	0.776	1.66e-19
PTLe00860	0.726	1.74e-14
PTLe00820	0.72	1.48e-12

Co-expressed WRKY genes are listed with Pearson correlation coefficient (r value > 0.7)

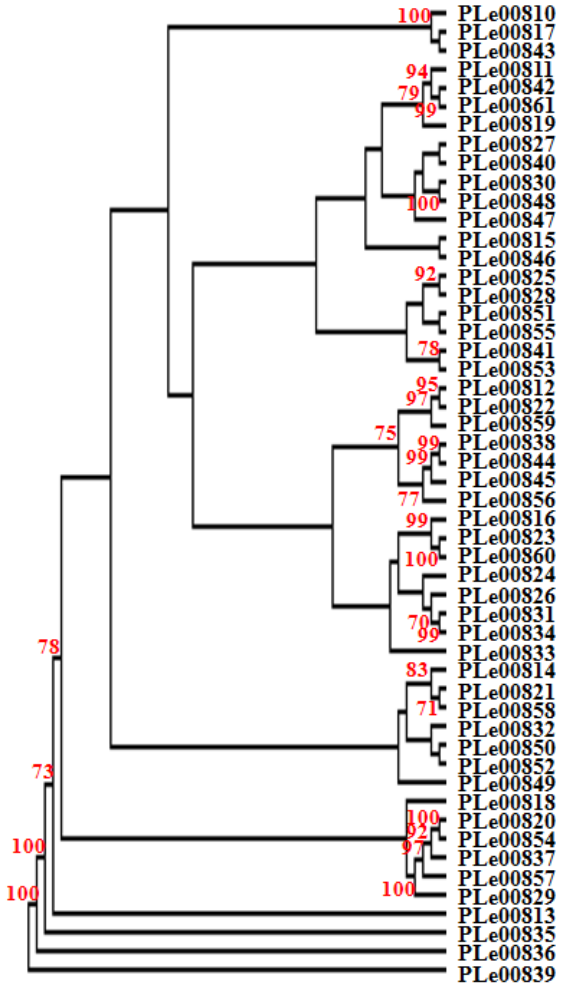

Fig 2. Phylogenetic relationships of tomato WRKY proteins. Phylogenetic analysis was carried out using the neighbor-joining method. Default values were used except for 100 bootstraps. Only bootstrap scores >70 are shown.

WRKY motif without zinc-finger, PTLe00814, PTLe00815, PTLe00821, PTLe00846, and PTLe00858 do not fall into subgroups (Fig. 2), which might be an indication that these WRKY proteins represent sequencing and assembly errors as suggested by Xie et al. (2005). Using co-expression data, the constitution of rice MAPK cascades has been predicted (Jung et al., 2010), indicating that the analysis of co-expression data is a useful tool for systematic functional analysis of large gene family members. Therefore, the Plant Expression Database was screened using tomato *WRKY*, *LeMPK2*, and *LeMPK3* mRNA sequences. The probe sets of *WRKY*, *LeMPK2*, and *LeMPK3* are listed in Table 1. As shown in Fig. 3, *LeMPK2* was constitutively expressed and not significantly affected by salt stress and *B. cinerea* infection. However, the strong induction of *LeMPK3* is observed during response to these stimuli. Similarly, it has been shown that *SIPK* (tobacco MAPK, homologous to LeMPK2) and *LpMPK2* are constitutively expressed, while the expression of *WIPK* (tobacco MAPK, homologous to LeMPK3) and *LpMPK3* are increased by wounding and other stresses (Zhang and Klessig 1998; Hyun et al., 2009). Fourteen out of 52 *WRKYs* were expressed by salt stress or/and *B. cinerea* infection (Fig. 3). The strong induction of *PTLe00820*, *PTLe00822*, *PTLe00854*, *PTLe00856*, and *PTLe00860* has been observed after treatment with salt and *B. cinerea*, whereas *PTLe00831*, *PTLe00834*, *PTLe00838*, *PTLe00843*, *PTLe00844*, and *PTLe00859* were highly expressed by salt treatment (Fig. 3). We then performed the co-expression analysis of *LeMPK3* gene from the Affymetrix genome arrays in 13 different experiments. After calculating correlating coefficients between gene-expression profiles for all tomato genes, we selected out *WRKY* genes with correlation coefficients of 0.7 or greater, and highlight them in Table 2. The strong co-expression between *LeMPK3* and *PTLe00820*, *PTLe00854* and *PTLe00860* might be indicated that these three

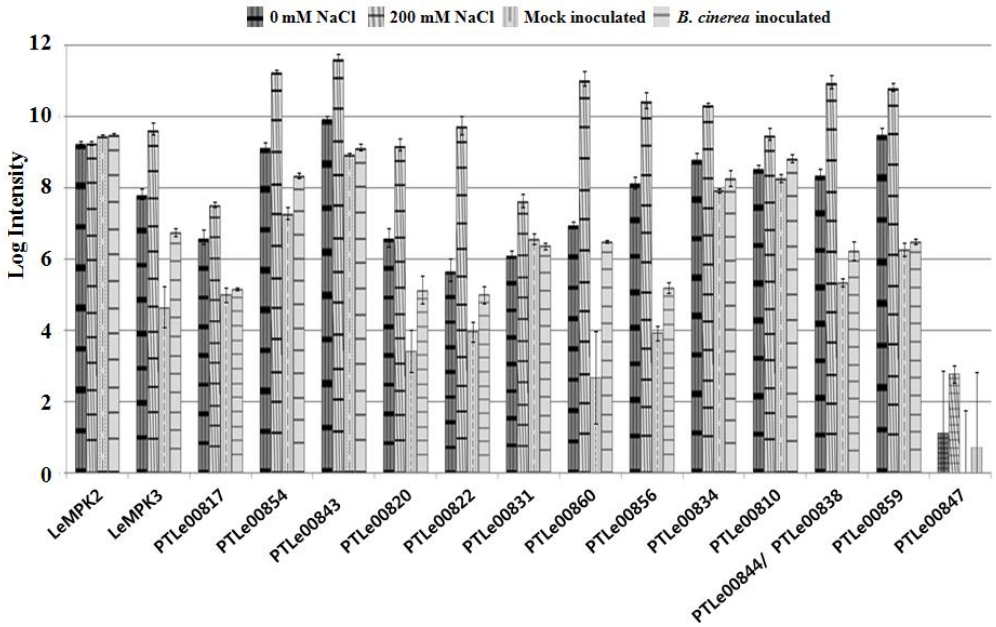

Fig 3. Transcript abundance of *LeMPK2*, *LeMPK3*, and *WRKY* genes during response to salt stress and *B. cinerea* infection. The values and error bars indicate the mean and standard error, respectively, from three independent hybridizations.

WRKYs are possible substrate of LeMPK3 during response to stress-related stimuli. MAPKs share a core set of effectors, but they also have specific roles that each particular MAPK plays as specific effectors to differentiate the response. These specific MAPK effectors seem to be related to specific substrates. In this study, we have identified three WRKY proteins as substrates of LeMPK3 during response to stress-related stimuli. Although further functional analysis of LeMPK3 and WRKYs through *in vivo* and *in vitro* experiments will be required, our study provides the basis for future research on the diverse signaling pathways mediated by MAPKs in tomato.

Material and methods

Plant material and stress treatment

Tomatoes (*S. lycopersicum* cv. Moneymaker) were grown under greenhouse conditions at 25°C with additional illumination of 60 klx from 07:00 to 19:00; plants that were 4-6 weeks old were used for the experiments. For induction of MAPK activation, leaves were infiltrated with 50 mM NaCl, or with 100 µg μL^{-1} PGA, or lightly pressed three times by a gloved hand (for wound stress). Samples for protein preparation were harvested at the time, snap frozen in liquid nitrogen, and stored at -80°C until analysis.

Protein extraction and immuno complex kinase activity assay

Proteins were extracted by grinding leaf tissue as described by Hyun et al. (2009). The concentration of protein extracts was determined according to the Bradford method (Bradford 1976) with bovine serum albumin as standard. The immuno-complex kinase assay was performed as described previously by Hyun et al. (2009). 1 µg of 4G10 antibody (Upstate, USA), 0.1% (v/v) Nouidet P-40 and 75 mM NaCl were added to 57 µL of protein extract (100 µg). After 1 hour on ice, about 20 µL of protein A-agarose (50% suspension, Oncogene, USA) was added, and the incubation on ice was continued for an additional 2 hours. The complexes were precipitated by a brief centrifugation and washed three times with a wash buffer (20 mM Tris, pH7.5, 5 mM EDTA, 100 mM NaCl, and 1% Triton X-100) and one time with a reaction buffer (200 mM HEPES, pH 7.5, 15 mM $MgCl_2$, 5 mM EGTA, and 1 mM DTT). 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 (MBP, Upstate, USA) and 1 µCi γ^{32}P-ATP. The phosphorylated MBP was visualized by autoradiography after being resolved on a 15% SDS-polyacrylamide gel.

In-gel kinase assay

Extracts containing 20 µg of total protein were loaded on 10% (w/v) polyacrylamide gels embedded with 0.3 mg mL^{-1} MBP in the separating gels as substrate for the kinase. After electrophoresis, proteins were renatured and assayed for kinase activity as described by Hyun et al. (2010). Activities were visualized by autoradiography and a phosphor imager.

In-silico analysis of tomato WRKY genes expression and phylogenetic tree construction

Fifty-two *WRKY* mRNAs were downloaded from Plant Transcription Factor Database, (http://planttfdb.cbi.pku.edu.cn:9010/index.php). For *in-silico* determination of expression of *WRKY* genes during response to salt stress and *Botrytis cinerea* infection, Plant Expression Database (PLEXdb, http://www.plexdb.org/index.php) was screened, using tomato *WRKY* mRNA sequences, by performing a bulk query. Co-expression analysis was performed in Tomato Functional Genomics Database (http://ted.bti.cornell.edu) using Affymetrix genome arrays. Co-expressed genes with *LeMPK3* were identified by calculating the Pearson correlation coefficients (r-values) with a cutoff value of 0.7. Phylogenetic analysis was carried out using the neighbor-joining method, and the phylogenetic tree was displayed using TreeTop

(http://www.genebee.msu.su/services/phtree_reduced.html).

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