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KNOCKOUT OF *ATMKK1* REDUCES *ARABIDOPSIS* RESPONSE TO 2, 3, 5-TRIIODOBENZOIC ACID IN LEAVES

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Abstract: Mitogen activated protein kinase (MAPK) pathways are crucial for plant growth and development. The most commonly identified pathways that AtMKK1 has been connected to are wounding, bacterial pathogen response, cold, drought, salt stress, reactive oxygen species stress, touch, and abscisic acid. There is also evidence that AtMKK1 regulates development. In leaf development, auxin can modulate both cell division and expansion and has a key role in both initiation and elaboration of final morphology of both leaves and vascular networks. Distribution of auxin to different tissues and organs relies on auxin transport systems. In our study, it was found that there was reduced response in *atmkk1*, the *AtMKK1* knockout mutant, to 2,3,5-triiodobenzoic acid, an auxin polar transport inhibitor. Analysis of protein-protein interactions has suggested that AtMKK1 may interact with the downstream AtMPK12, which is a negative regulator of auxin signaling. Our results indicate that AtMKK1 may play a role in leaf development.

Keywords: *Arabidopsis*, auxin, leaf, PIN1, TIBA.

Introduction

Mitogen-activated protein kinase (MAPK) pathways play an important role in regulation of plant growth and development. These pathways consist of at least three core enzymes: a MAPK (MPK), activated by a MAPK kinase (MAPKK, MKK or MEK), which is in turn activated by a MAPK kinase kinase (MAPKKK, or MEKK). MAPKKs all have a common activation motif, S/TXXXXXS/T, as well as a high specificity for the downstream MAPKs [MAPK Group., 2002; XING & al. 2002]. In *Arabidopsis* there are approximately 20 MAPKs, 10 MAPKKs, and about 12-60 MAPKKKs [XU & ZHANG, 2015]. The functions of most of them are unknown. There are several completed pathways that have been identified, but the relationships between many MAPK cascade proteins are still not clear. While all components in a MAPK cascade have the ability to phosphorylate and activate their direct downstream targets, MAPK cascade proteins can also be involved in pathways without direct interaction through activating other pathways or by acting as scaffold proteins [MESZAROS & al. 2006]. The cross talk and wide ranging interactions of MAPK pathways also mean that the isolation of a single point of effect from the removal or overexpression of a gene is extremely difficult.

Some fully identified pathways are as follows. AtMEKK1-AtMEK1/AtMEK2-MPK4, involved in a variety of stresses ranging from pathogen response, to wounding, to environmental stresses such as salt or temperature [PITZSCHKE & al. 2009; QIU & al. 2008; TEIGE & al. 2004]. The most convincing and representative work in the identification of a complete signaling path was from the analysis of *Arabidopsis* response to flagellin, a highly conserved component of bacterial flagella that functions as a pathogen-associated molecular

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pattern (PAMP) in plants and mammals [ASAI & al. 2002]. The signaling components include a complete plant MAP kinase cascade (MEKK1, MKK4/MKK5 and MPK3/MPK6) and WRKY22/WRKY29 transcription factors that function downstream of the flagellin receptor FLS2, a leucine-rich-repeat (LRR) receptor kinase in *Arabidopsis* [ASAI & al. 2002]. The majority of study on MAPK cascades in *Arabidopsis* has been carried out in stress response analysis [COLCOMBET & HIRT, 2008], but there are examples of MAPK pathways in *Arabidopsis* playing roles in developmental cascades, such as dwarfism seen in *mpk4* and *mekk1* mutants [QIU & al. 2008], or changes in stomata patterning or pollen development [COLCOMBET & HIRT, 2008].

A complete pathway for AtMKK1 has been identified, AtMEKK1 was shown to phosphorylate AtMKK1 [HADIARTO & al. 2006], and activate AtMKK1 in a yeast two hybrid system. AtMPK4 was shown to be connected to AtMKK1 in the same yeast two hybrid system [MIZOGUCHI & al. 1998]. Since the initial identification of the AtMEKK1-AtMKK1-AtMPK4 pathway a multitude of other interactors have been identified. AtMKK1 plays a role in the regulation of not only AtMPK4, but also AtMPK3 and AtMPK6 [MESZAROS & al. 2006; XING & al. 2008]. The upstream AtMEKK1 can also activate numerous downstream proteins such as AtMKK2, AtMKK4, AtMKK5, and the downstream targets of AtMKK1 can also be activated by AtMKK2, AtMKK4, and AtMKK5 [COLCOMBET & HIRT, 2008]. The most commonly identified pathways that AtMKK1 has been connected to are wounding, bacterial pathogen response, cold, drought, salt stress, reactive oxygen species (ROS) stress, touch, and abscisic acid (ABA) [CONROY & al. 2013; HADIARTO & al. 2006; MATSUOKA & al. 2002; MESZAROS & al. 2006; PITZSCHKE & al. 2009; TEIGE & al. 2004; XING & al. 2008]. Some of the stresses such as wounding have been accepted with little in the way of conflicting data, but other stresses such as salt have met with mixed responses. There are studies linking AtMKK1 with the activation of MPK4 in salt stress [MATSUOKA & al. 2002], but there are other studies that have refuted that claim and stated that AtMKK1 is not involved in salt response [TEIGE & al. 2004]. Our previous work has indicated that AtMKK1 knockout mutant, *atmkk1*, could germinate in highly saline environments to a level far above that of the wild type, and the adult plants could resist the effect of high salinity, indicating that AtMKK1 is likely a negative regulator of salt stress response [CONROY & al. 2013]. Studies have indicated that that AtMKK7 controls plant architecture through the negative regulation of polar auxin transport (PAT) [DAI & al. 2006] and AtMPK12 is a negative regulator auxin signaling [LEE & al. 2009]. However, little is known about the involvement of AtMKK1 in auxin signaling pathways [XU & ZHANG, 2015].

Auxin can modulate both cell division and expansion, and has a key role in both initiation and elaboration of final morphology of both leaves and vascular networks [SCARPELLA & al. 2010]. A key feature of auxin action is the existence of feedback loops through which auxin regulates its own transport [BAYER & al. 2009; GUENOT & al. 2012; HEISLER & al. 2005; WISNIEWSKA & al. 2006]. AtMPK12 acts as a negative regulator of auxin signaling, with IBR5, a protein phosphatase that targets AtMPK12 [LEE & al. 2009]. Some MAP kinase cascade components are also shown to function in rosette leaf expansion (e.g. MAP3Ke1/MAP3Ke2) [CHAIWONGSAR & al. 2012] and cell plate expansion (AtMPK4 and AtMPK11) [KOSETSU & al. 2010]. More recently, a genome-wide search of the rice genome database and a yeast two-hybrid assay identified OsAux/LAX1, an auxin influx carrier, as a potential target protein of OsMPK3, OsMPK4 and OsMPK6, suggesting

a direct involvement of MPKs in the auxin transport and signaling pathway [MOHANTA & al. 2015]. Highly specific and transient expression of AtMPK10 determines auxin-induced leaf venation patterns in *Arabidopsis* and the AtMKK2-AtMPK10 module regulates venation complexity by altering polar auxin transport efficiency [STANKO & al. 2014]. Here, leaf phenotypic changes were analyzed after *atmkk1* plants were treated with 2, 3, 5-triiodobenzoic acid (TIBA), an auxin transport inhibitor.

Materials and methods

Mutant plants and plant growth conditions

T-DNA insertion mutant was obtained from the ARBC (The *Arabidopsis* Resource Centre; http://www.*Arabidopsis*.org). Details on its locus identification, insertion confirmation and selection on selective media were described previously [CONROY & al. 2013]. Seeds were surface sterilized, stratified for 4 days and then plated on MS plates containing kanamycin. Wild type (WT) seeds were not plated on plates containing kanamycin. Mutant (line A51) and wild type *Arabidopsis* (Col-0) were grown in a growth chamber (ENCONAIR Technologies Inc.) under a 16 h light and 8 h darkness cycle at 22 °C. After ten days the seedlings were transferred into autoclaved soil and allowed to grow for a further fourteen days before experimental treatments began.

Response to TIBA

Treatments was carried out using 2, 3, 5-triiodbenzoic acid (TIBA) at a concentration of 0.1 mM (first dissolved in ethanol and then made to the concentration with H_2O). The solution of TIBA was spread over leaves using a cotton swab with a water control. Leaf width and length were measured at 0 and 6 days. Leaves from both time points were also frozen in liquid N_2 and stored at -80 °C till further use.

RT-PCR

RT-PCR was used for the evaluation of PIN1 expression with primers 5'-TGCAGGTCTAGGCATGGCTA-3' and 5'-TTTAACGCCATGAACAACCCA-3'. Actin2 primers used are 5'-CCTCATGCCATCCTCCGTCTTG-3' and 5'-TTCCATCTCCTGCTCGTAGTCAAC-3'. PCR was carried out under the following conditions: 94 °C for 3 min; 30 s at 94 °C, 30 s at 59 °C, and 30 s at 72 °C for 25 cycles; and then 10 min at 72 °C.

Bioinformatics analysis

Protein-protein interactions were predicted using STRING 9.0 (http://string-db.org/) databases.

Results

Effect of TIBA on leaf growth

The effect of TIBA, an auxin transport inhibitor, on leaf growth was examined. Leaf length and width were inhibited in wild type but not in *atmkk1* plants (A51) (Fig. 1). The ability to grow in the wild type leaves is significantly suppressed after the addition of the TIBA, contrast that to the unchanged parameters of the *atmkk1* plants (Fig. 1). The *atmkk1* plants appeared to be capable of resisting the effect of the TIBA more dramatically than the wild type.

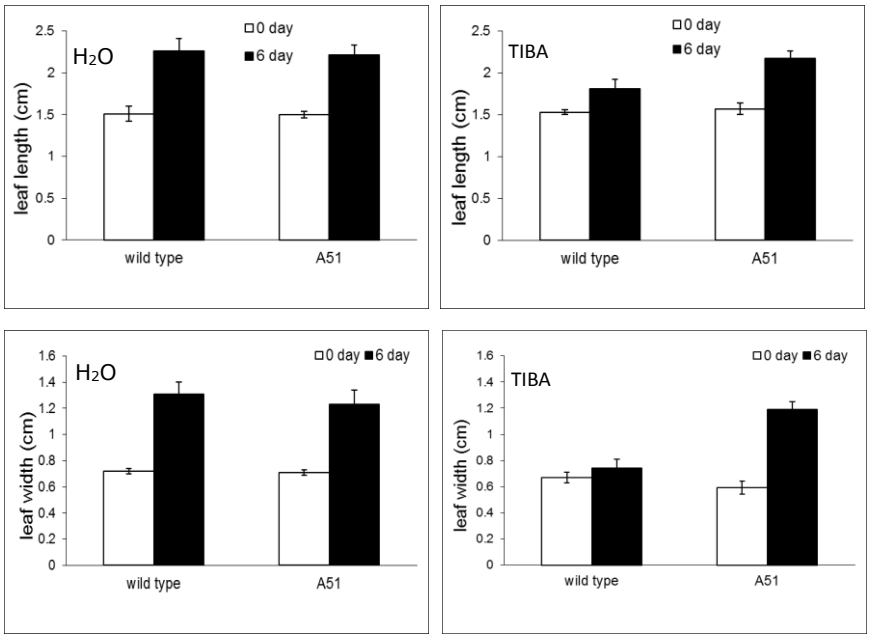

Fig. 1. Leaf length and width after TIBA treatment. Leaves were treated with TIBA for 6 days. This experiment was carried out three times.

Expression of PIN1 upon TIBA treatment

Alterations of auxin transport could induce changes in PIN gene expression [BLAKESLEE & al. 2004]. We examined the expression level of PIN1 after TIBA treatment. While in wild type the expression level was increased by about 8 fold, PIN1 expression was slightly inhibited in *atmkk1* (A51) leaves (Fig. 2). However, the knockout mutant seems to have a background PIN1 level that is 5-6 fold higher than that in the wild type (Fig. 2). It suggests that the knockout of *AtMKK1* may have enhanced PIN1 expression.

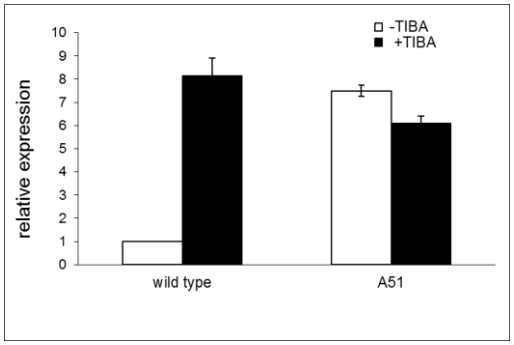

Fig. 2. Expression of PIN1 after TIBA treatment. This experiment was carried out three times.

Prediction of interactive partners of AtMKK1

Protein-protein interaction analysis with STRING indicates that AtMKK1 interacts with AtMPK4, 11, and 12 (Fig. 3 and Tab. 1). AtMPK12 is a negative regulator of the auxin transduction signaling pathway [LEE & al. 2009].

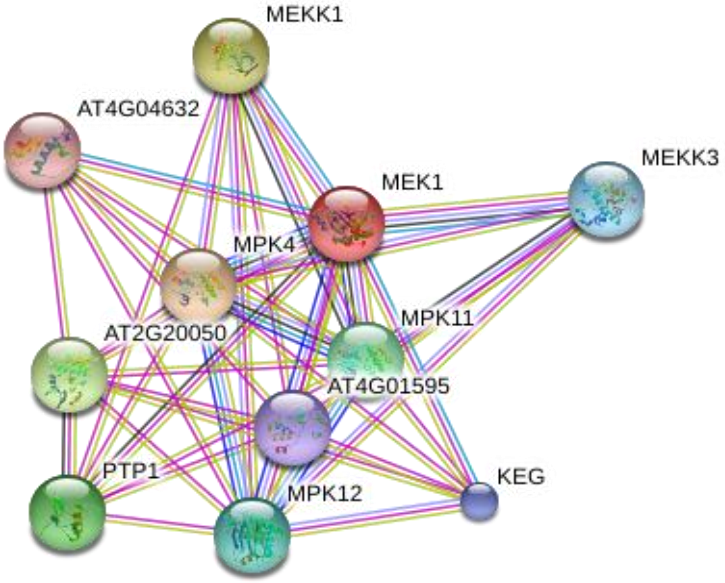

Fig. 3. Prediction of interactive partners of AtMKK1.

Tab. 1. Possible functional partners of MAP kinase kinase 1. The prediction tool STRING 9.0 (http://string-db.org/) used different modes of prediction to determine which proteins interact with MAPKK1 and described most of their associated functions.

Protein ID/ Name	Mode of prediction	Function
MPK4 MAP kinase 4	Experiments, textmining, homology, co-occurrence, co-expression, databases	Involved in cortical microtubules organization and stabilization. Involved in root hair development process. Negative regulator of systemic acquired resistance (SAR) and salicylic acid- (SA) mediated defense response. Required for jasmonic acid- (JA) mediated defense gene expression. May regulate activity of transcription factor controlling pathogenesis-related (PR) gene expression. Seems to act independently of the SAR regulatory protein NPR1 (Nonexpresser of PR1).
MEKK1 MAPK/ERK kinase kinase 1	Experiments, textmining, homology, databases	Involved in the innate immune MAP kinase signaling cascade (MEKK1, MKK4/MKK5 and MPK3/MPK6) downstream of bacterial flagellin receptor FLS2. May be involved in the cold and salinity stress-mediated MAP kinase signaling cascade (MEKK1, MEK1/MKK2 and MPK4/MPK6). Activates downstream MKK2, MKK4 and MKK5
AT2G20050	Experiments, textmining	protein phosphatase 2C and cyclic nucleotide-binding/kinase domain-containing protein

PTP1 tyrosine phosphatase 1	Experiments, textmining, co-expression	Protein-tyrosine-phosphatase that dephosphorylates and probably inhibits MPK6 in non-oxidative stress conditions. In association with MKP1, represses salicylic acid (SA) and camalexin biosynthesis, thus modulating defense response. May also repress MPK3. Dephosphorylates and inactivates MPK4 in vitro
MPK11 MAP kinase 11	Experiments, textmining, homology, co-occurrence, co-expression	
MPK12 mitogen-activated protein kinase 12	Experiments, textmining, homology, co-occurrence	Negative regulator of the auxin transduction signaling pathway
MEKK3 MAPK/ERK kinase kinase 3	Experiments, textmining, homology, co-expression, databases	
KEG- KEEP ON GOING	Experiments, textmining, databases	Mediates E2-dependent protein ubiquitination. Acts as a negative regulator of abscisic acid signaling. Required for ABI5 degradation, by mediating its ubiquitination. Together with EDR1, may regulate endocytic trafficking and/or the formation of signaling complexes on trans-Golgi network (TGN)/ early endosome (EE) vesicles during stress responses
AT4G01595 *protein kinase-like protein*	Experiments, textmining	
AT4G04632- protein kinase family protein	Experiments, textmining, databases	

Discussion

Experiments carried out using TIBA may indicate that AtMKK1 could be involved in auxin response pathways. The data show a trend towards there being less impact upon the AtMKK1 mutation following TIBA treatment. TIBA had a different effect upon the growth of the leaves. While there was a noticeable difference in the leaf growth of the wilt type leaves, there was little change in the response of the AtMKK1 mutation. The *atmkk1*plants appeared to have the same growth patterns regardless of whether TIBA was applied to the leaves or not. The treatment showed that alerting the auxin transport in the leaves of wild type plants can have a significant impact upon the growth patterns. But the inhibition of polar auxin movement had little effect on *atmkk1* plants. The *atmkk1*plants were phenotypically indifferent to TIBA (data not shown). Whether AtMKK1 mutations allow for *Arabidopsis* plants to resist the changes of auxin transport, or just better regulate the movement of auxin in the leaf blades is unknown.

ABP1 had been considered a membrane-bound auxin receptor until recent re-analysis of new *abp1* null alleles generated by CRISPR/Cas9 [GAO & al. 2015]. The loss of ABP1 resulted in no obvious defects in auxin response and *Arabidopsis* morphology [GAO & al. 2015]. It is suggested that ABP1 is likely not the auxin receptor for auxin-mediated non-genomic effects and it is worth revisiting the hypothesis that auxin efflux carriers may serve as auxin receptors [GAO & al. 2015; STRADER & ZHAO, 2016]. Root, young leaf and seedling growth are found to be regulated by PAT. PIN (PIN-FORMED) proteins are well-characterized auxin efflux carriers, playing essential roles in many developmental processes [GALWEILER & al. 1998; PETRASEK & al. 2006]. Genetic studies suggest that

members of several gene families such as PID (PINOID), NPY (NAKED PINS IN YUC MUTANTS), and ARF (AUXIN RESPONSE FACTOR) can potentially function downstream of PIN1. PIN and PID may form a plasma membrane localized auxin receptor complex important for auxin-mediated *Arabidopsis* organogenesis. As PID lacks a receptor domain, it is hypothesized that PIN proteins may function as an auxin receptor based on genetic evidences. Previous studies focused on the capacity and directionality of PIN-mediated auxin transport. This new model suggests that auxin transport may be coupled with a signal transduction pathway, which can reasonably account for the observed pin-like phenotypes in various *Arabidopsis* mutants [STRADER & ZHAO, 2016]. TIBA was also shown to have only slight effects on PIN1 localization on their own and leave PIN1 accumulation at the plasma membrane unaffected [GELDNER & al. 2001]. Genevestigator data mining also indicated that TIBA treatment (3 hours) did not have effect on AtMKK1 or PIN1 expression in *Arabidopsis* seedlings (data not shown), while TIBA treatment for 6 days in our work enhanced PIN1 expression level in wild type *Arabidopsis*.

In the attempt to find possible mechanisms, we examined if AtMKK1 knockout may lead to disconnection of components in MAP kinase cascade. Search for potential AtMKK1 interactive proteins has indicated that the expression of AtMPK4, 11, and 12 co-occur and co-expressed with AtMKK1, and particularly each of these three AtMPKs interacted with AtMKK1experimentally (Tab. 1). The analysis also indicates that AtMKK1 experimentally interacted with upstream MEKK1 and MEKK3. AtMKK7 was shown to mediate the regulation of auxin polar transport [DAI & al. 2006] and at the MPK level, there is evidence that AtMPK12 is a negative regulator auxin signaling [LEE & al. 2009]. MAPK cascade involvement in PAT is also shown in previous studies. Increased expression of AtMKK7 caused deficiency in polar auxin transport and leads to plant architectural abnormality in *Arabidopsis* [DAI & al. 2006]. It was thus suggested that AtMKK7 negatively regulates PAT. It is possible that loss of connection of AtMKK1 to AtMPK12 in *atmkk1* plants may be attributed to reduced response to auxin fluctuation upon TIBA treatment.

Conclusions

AtMKK1 is a protein involved in *Arabidopsis* defense and developmental pathways. While the literature has presented numerous, often contradictory roles for AtMKK1, this project has provided some clarification of the uncertainties surrounding AtMKK1, as well as providing new potential areas for research. The fact that *atmkk1*plants were unaffected by TIBA while the wild type did show signs of impact allows for the conclusion that AtMKK1 may play a role in auxin signaling. The possible targets for AtMKK1 in auxin signaling remain unclear and the precise role of AtMKK1 in auxin signaling should be further studied. Among these, it would be interesting to study if and how AtMKK1, PIN1, and the regulation of auxin responsive genes are connected, and if PIN1 protein is modified at post-translational level when connection of AtMKK1 and AtMPK12 is lost due to the knockout of *AtMKK1*.

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