

INVESTIGATING PROTEIN PARTNERS OF ATMKK1 AS PART OF THE MAPK SIGNALING PATHWAY DURING SALT STRESS

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Abstract: Mitogen-activated protein kinase cascades are one of the many systems that allow plants to survive and defend themselves against pathogens and other environmental stresses. Numerous scientific investigations rendered insights to molecular signaling pathways that take place in an event of a stress such as soil salinity. Despite the known functions and locations of proteins that play a role in these pathways, very little is known about upstream protein partners. In this paper, we elucidate biological functions and molecular locations of *Arabidopsis thaliana* MKK1 protein through data mining predominantly from STRING and BAR databases. Results revealed AtMEKK1 and CRLK1 as upstream protein partners. In addition, AtMKK2 was further analyzed as a redundant protein to AtMKK1.

Keywords: *Arabidopsis*, AtMEKK, AtMKK, CRLK, protein interaction, salt response.

Introduction

The plant kingdom is one of the eukaryotic domains that hugely contribute to oxygen, food, industrial, and pharmaceutical productions. Aside from photosynthetic capabilities, plants are also characterized for their sessile nature. Unlike animals, plants cannot migrate when environmental conditions become intolerant. Instead, they have elaborate systems that enable success and compatibility in a specific habitat. One of these systems is mitogen-activated protein kinase (MAPK) cascades, which are modules that operate through signal transduction in response to environmental and endogenous stimuli. MAPK cascades are crucial in plant growth, development, and more importantly, defense against stresses. Some of the known stresses from which plants are subjected to are pathogens, oxidative stress, extreme temperatures, high salinity, wounding, osmolarity, etc. [ZHANG & KLESSIG, 2001]. The MAPK signaling pathway responds to these threats by either positively or negatively regulating various elements in signal transduction [XING & FOROUD, 2021]. Initiation of the pathway starts with ligand binding to cellular receptors. For an instance, following a pathogen attack, conserved molecules derived from the pathogen called pathogen-associated molecular patterns (PAMPs) interact with plant pattern receptors [PITZSCHKE & al. 2009; XING & FOROUD, 2021]. Then in the downstream signaling process, MAP kinase kinase kinase (MAPKKK or MEKK) activates MAP kinase kinase (MAPKK or MKK) which also then activates MAP kinase (MAPK) [XING & FOROUD, 2021]. The diversity of kinases offers a wide range of activation combinations. And with further complexity brought upon by protein interactions at various levels, plants are able to possess an elaborately versatile system against environmental stresses [HAMEL & al. 2006].

The relay of phosphorylation in MAP kinase cascade is a crucial step in plant signal transduction. Protein phosphorylation is one of the many regulatory processes which takes place in the cellular and molecular level following an exposure to abiotic stress [KUMAR & al. 2020].

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In the case of MAPKs, MAPKKs transfer phosphate groups to MKKs' activation motifs, serine and/or threonine residues. Then, MKKs phosphorylate threonine and tyrosine (T-X-Y) residues of downstream MAPKs. Once activated, MAPKs further phosphorylate downstream proteins [KUMAR & al. 2020].

Arabidopsis thaliana is a flowering plant that is frequently used in plant biochemistry and molecular genetic investigations. It has been found that *Arabidopsis* has approximately 80 MAPKKs, 10 MKKs, and 15-20 MAPKs [XING & FOROUD, 2021]. These divergent protein kinases offer a wide variation of combinations in signal transduction. It is important to note, however, that a singular pathway is not unique to one specific stress stimuli. Multiple stresses can trigger activation of the same pathway. For example, the AtMEKK1-AtMKK1/AtMKK2-AtMPK4 pathway can be activated in response to both pathogen attack and salt stress stimuli [CONROY & al. 2013]. This is due to the fact that activation of a specific kinase can actually result in phosphorylation of multiple kinases that are involved in various other pathways. Although identification of signaling pathways has immensely aided in a deeper elucidation of downstream regulatory processes, numerous cascade genes still have unknown functions [CONROY & al. 2013]. In addition, most of the identifications are centred towards downstream proteins but there are limited available insights on upstream protein partners [TEIGE & al. 2004].

In this paper, we elucidate the functional role that AtMKK1 plays in response to salt stress. Through data mining and network analysis, upstream protein partners of AtMKK1 were identified to advance the understanding on cellular and molecular interactors associated in salt response pathways.

Material and methods

Protein-protein interaction partners

Identification of protein partners of AtMKK1 was carried out using the STRING (<https://string-db.org>). STRING is a database that offers a visual network of protein-protein interactions through physical and functional relations. Currently, the database renders computational network predictions of more than 24 million proteins and 5,000 organisms. Input of the AtMKK1 under the protein name search tab autodetected several matches from 41 organisms, one of which was *Arabidopsis*. Yielded network prediction primarily exhibited query proteins and first shell of interactors associated to AtMKK1. Each predicted partner was analyzed for its function and position relative to AtMKK1 through the information provided on the database itself. Moreover, additional analysis of the interactors was also carried out through redirection from STRING to AlphaFold Protein Structure Database (<https://alphafold.ebi.ac.uk>). This artificial intelligence (AI) system offers protein information about the gene as well as its quaternary structure. Biological functions of the proteins of interest as well as the signaling pathways of which they play a role in are gathered from AlphaFold. Twelve primary protein partners of AtMKK1 were identified and yielded network of predicted interactors was then screen captured from the STRING site.

Co-localization and co-expression

Co-localization and co-expression of upstream protein partners of AtMKK1 were elucidated using The Bio-Analytic Resource (BAR) for Plant Biology website by the University of Toronto (<http://bar.utoronto.ca>). BAR is a bioinformatic site that offers web-based tools which are mostly centred on genomics and protein-protein interactions. The portal also displays

visualizations of gene expression in 15 plant species. To analyze the three target protein partners, the ePlant browser under Gene Expression and Protein tool was launched. Genes AtMEKK1 (AT4G08500), AtMKK2 (AT4G29810), and CRLK1 (AT5G54590) were entered into the search tab, revealing informative viewers that range from description about the genes to their sequence data. Co-localization of each gene was analyzed in organ, tissue, and cellular levels through a linear score of gene expression. A red colorization of an organ, tissue, and organelle is indicative of high gene expression while bright yellow suggests a linear score of 0, hence no gene expression. On the other hand, co-expression of the genes was identified under the Tissue Specific Root eFP and Abiotic Stress eFP. These particular viewers render visualization of gene expression in specific root tissues as well the plant shoot in response to salt stress.

Data Analysis

Values of gene expression were obtained from the BAR database. In this study, co-expression of the target genes in the shoot and root after salt treatment is shown in scatter plots with smooth lines generated from Microsoft Excel software.

Results

Protein-protein interactions

Twelve proteins were identified as first shell of interactors to AtMKK1 through the STRING database (Figure 1). Two proteins, MEKK1 and CRLK1, were also determined to be upstream protein partners of AtMKK1. Identification of biological function through the AlphaFold database revealed that AtMEKK1 participates in the negative regulation of innate immunity as a defense against pathogens. The protein's location, relative to AtMKK1, is upstream based on the fact that MAPKKs function upstream and activate downstream MAPKKs [KONG & al. 2012; XING & FOROUD, 2021]. Next, recognition of calcium/calmodulin-regulated receptor-like kinase 1 (CRLK1) as a protein partner of AtMKK1 was carried out independently from the STRING database. As shown in Figure 1, CRLK1 was not established as a primary partner nor was it part of the second shell of interactors. Identification of the function of AtMEKK1 through the AlphaFold site rendered redirection to the Uniprot website which associated a relevant publication about the gene. The article revealed that CRLK1 phosphorylates AtMEKK1 in response to cold stress [FURUYA & al. 2013]. This phosphorylation is indicative of the fact that CRLK1 functions upstream of AtMEKK1 and is therefore also a further upstream regulator of AtMKK1. Lastly, among the first shell of interactors, MKK2 has been previously shown to function redundantly with AtMKK1 in activating the MPK4 pathway [PITZSCHKE & al. 2009]. Through the STRING and AlphaFold databases as well as applicable publications, we identified AtMEKK1, CRLK1, and AtMKK2 as our target genes in investigating upstream protein partners and other equally relevant interactors to AtMKK1 as part of the salt stress signaling pathway.

Co-localization

Co-localization of the three target genes in this study was identified through recognition of their expression in organ, tissue, and cellular levels. Expression of target genes was measured through quantified gene expression levels (GEL) aided by data visualizations. AtMEKK1 is highly expressed in dry seed (216.37), senescent leaf (184.05), cauline leaf (127.5), rosette leaf 4 (131.1), and rosette leaf 2 (124.03) (Figure 2A). On the other hand, high levels of AtMKK2 expression are found in most of the plant's leaves (Figure 2B), with a maximum expression localized in the

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proximal half of leaf 7 (400.1). Moreover, the pedicel (322.62) and the sepal (395.77) of flower stage 15 also highly express the AtMKK2 gene. Figure 2C, on the other hand, exhibits high levels of CRLK1 expression (maximum score: 64.52) in all stages of shoot apex, second internode of the stem (49.92), and the entire rosette after transition to flowering (47.7). At this level, AtMEKK1 and AtMKK2 are both highly localized in cauline leaf, senescing leaf, and rosette leaves 4 and 7 while AtMKK2 and CRLK1 co-localize the entire rosette after flowering. Among the three genes, it is apparent that AtMKK2 has the highest expression level while the GELs for CRLK1 range between 0 and 64.5.

At the tissue level, tissues of the root and shoot apex were analyzed. Tissue specific root eFP of the AtMEKK1 gene (Figure 3A) exhibits the highest level of expression in the endodermis (270.28) and phloem pole pericycle (270.23) of the root's elongation zone. Moderately consistent expression of the gene in the endodermis in all zones of the root is also apparent. AtMKK2, however, displays a different expression level pattern (Figure 3B). Highest level of AtMKK2 expression is observed in the columella of the meristematic zone (1637.7). Moderate levels of expression are centred around root tissues of the maturation zone. CRLK1 is highly expressed in the lateral root cap (331.81) of the apical meristem (Figure 3C) and moderate expression levels are seen in the xylem (133.08) of the zone of elongation. Neither of the three target genes share common sites of localization as increased expression of each are distributed in different zones of the root. In the shoot apex (Figure 4), the gene expression level of AtMEKK1 is highest in the leaf abaxial (FIL) (18.71) followed by the rib meristem (WUS) (16.55) while AtMKK2 is highly expressed in the epidermal 1 layer (TML1) (26.98) then in the enlarged peripheral zone (UFO) (24.95). AtMKK2 share a similar level of gene expression with CRLK1 as the gene is also highly expressed in the UFO (5.07). However, its highest level of expression is in the leaf adaxil (AS2) (6.21).

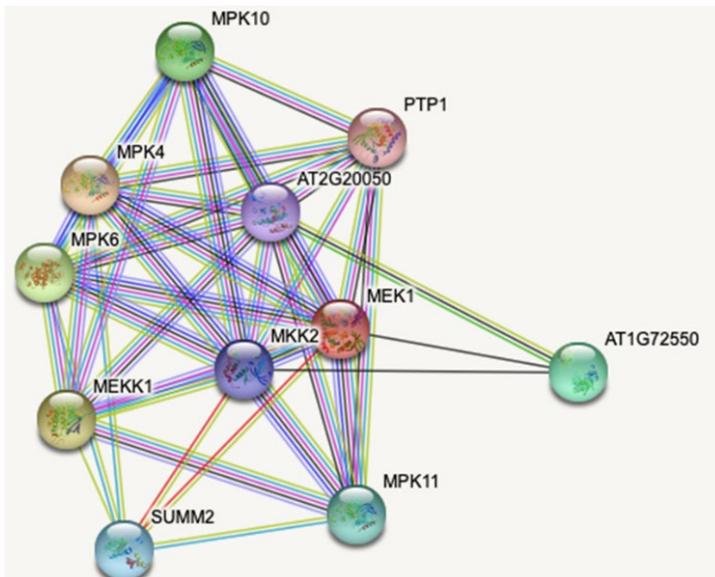


Figure 1. Protein-protein interaction network of the mitogen-activated protein kinase kinase 1 (MKK1) in *Arabidopsis thaliana*. Visual network representation was screen captured from the STRING database.

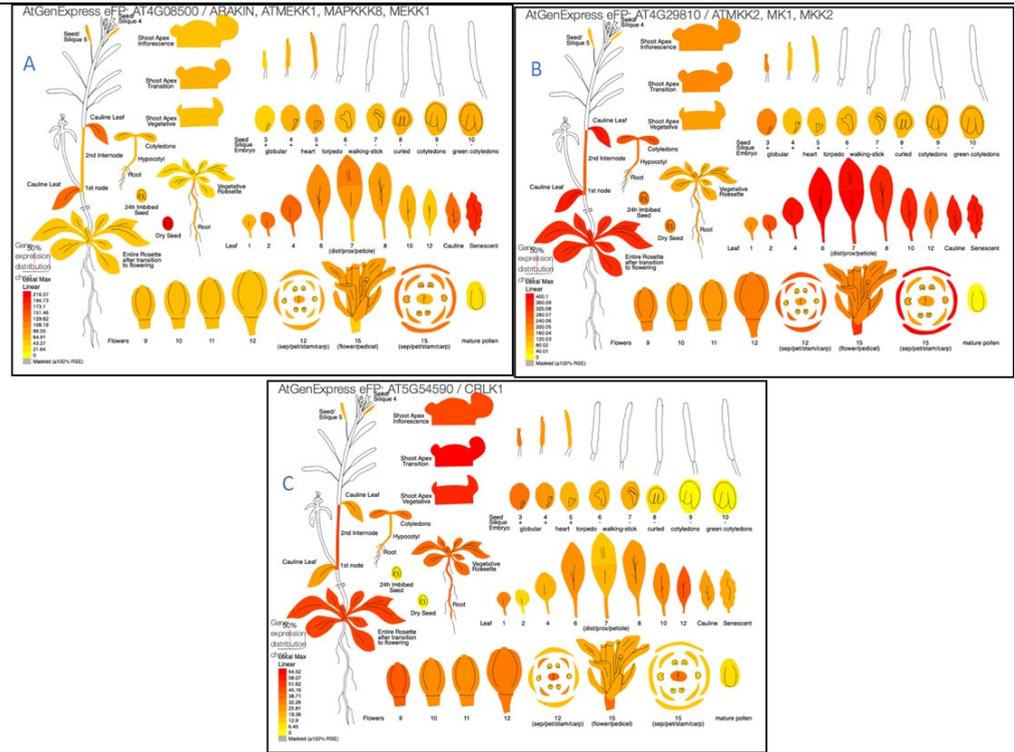


Figure 2. Localization of (A) MEKK1, (B) MKK2, and (C) CRLK1 in *Arabidopsis thaliana* at the organ level. Red colouration is indicative of high levels of gene expression while bright yellow represents absence of gene expression. Visualization was generated from the Plant eFP viewer of the BAR database.

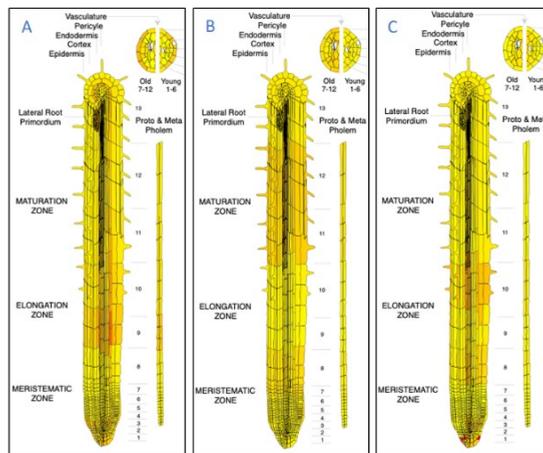


Figure 3. Localization of (A) MEKK1, (B) MKK2, and (C) CRLK1 in *Arabidopsis thaliana* in root tissues. Red colouration is indicative of high levels of gene expression while bright yellow represents absence of gene expression. Visualization was generated from the Tissue Specific Root eFP viewer of the BAR database.

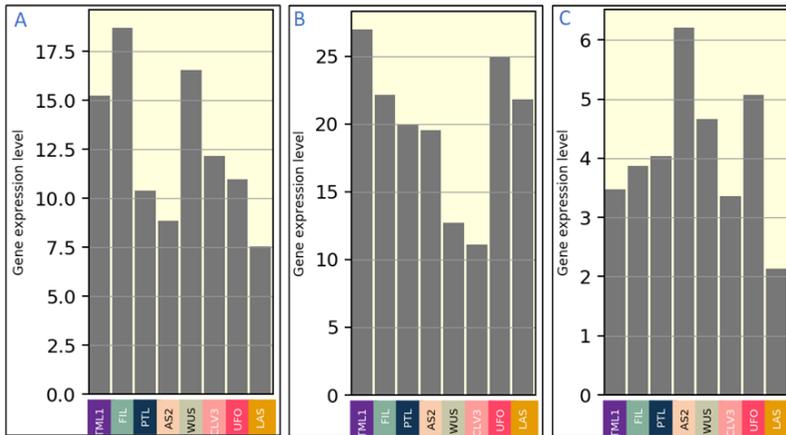


Figure 4. Gene expression levels of (A) MEKK1, (B) MKK2, and (C) CRLK1 in *Arabidopsis thaliana* in various zones of the shoot apex. Charts of expression values were obtained from *Arabidopsis* eFP Browser of the BAR database.

At the cellular level, AtMEKK1 is only highly expressed in the nucleus (32) (Figure 5A). Most of the organelles do not express the gene, with certain exception to the mitochondrion (8) and cytosol (8) for moderate GEL. AtMKK2 exhibits the opposite expression pattern as most of the subcellular locations do express the gene. Highest GEL is in the cytosol (20) while moderate levels are in the vacuole (10), Golgi body (10), mitochondrion (8), and nucleus (6) (Figure 5B). Lastly, CRLK1 is highly expressed in the Golgi body (14), endoplasmic reticulum (14), extracellular membrane (14), and plasma membrane (8) (Figure 5C). Moderate levels of GEL are in the nucleus (6) and cytosol (4). At this level, both the AtMKK2 and CRLK1 are highly expressed in the Golgi body and moderate expression of these genes are also seen in the nucleus. AtMEKK1 and CRLK1 do not share any cellular co-localization, however.

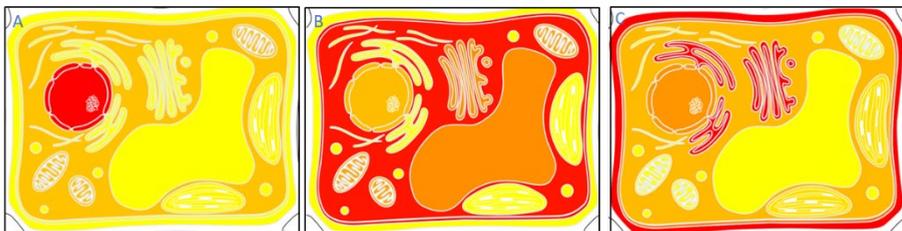


Figure 5. Gene expression levels of (A) MEKK1, (B) MKK2, and (C) CRLK1 in *Arabidopsis thaliana* at the subcellular level. Red colouration is indicative of high levels of gene expression while bright yellow represents absence of gene expression. Images were generated from Cell eFP viewer of the BAR database.

Co-expression

Co-expression of the three target genes was identified through BAR's Abiotic stress eFP and *Arabidopsis* eFP browser. In the experiment concerning each gene, *Arabidopsis* was grown under environmental conditions with 150 mM salt (NaCl). Gene expressions were measured at seven varying times with hour 0 and hour 24 post-exposure. Expressions of the

genes in the shoot and root are visually represented (Figure 6). Starting with AtMEKK1, at hour 0, GEL in the shoot is 54.59 while GEL in the root is 54.52. Gene expression levels in both the root and the shoot immediately increased 30 minutes after salt exposure, however, past 0.5 hour, patterns of expression levels varied between the two. Expression of AtMEKK1 was at its highest 1-hour post salt exposure then decreased until hour 6 where expression levels went back up to baseline (Figure 7A). After 24 hours, GEL in the shoot was 59.64. AtMEKK1 expression levels in the root decreased after hour 1 but a sudden increase is seen at hour 6 where a maximum GEL of 249.75 was reached. Past the hour of 6, expression levels decreased and a GEL of 144.68 was recorded after 24 hours post-treatment. Expression levels of the AtMKK2 (Figure 7B) follow a similar pattern as AtMEKK1. At hour 0, the shoot has a GEL of 151.77 which increased after an hour of salt treatment. Following a decrease at hour 3, GEL increased until a maximum level of 244.43 was achieved at hour 12. In the root, AtMKK2 expression level at hour 0 was 205.61. An increasing trend can be seen after salt exposure up to a maximum level of 432.75 at hour 6. Baseline levels were achieved as GEL decreased and after 24 hours, recorded data was at 264.49. Expression levels of CRLK1 in *Arabidopsis* (Figure 7C) in response to salt stress is varied among the other two target genes. In the shoot, the GEL at hour 0 started off at 53.81, which was the highest recorded level within the 24-hour period. From here, GEL had decreased until it hit its lowest value at hour 6 (37.52) then proceeded to increase at hour 12 (49.7). A GEL of 29.12 was recorded 24 hours post-treatment. In the root, the value at hour 0 was 31.93 which immediately increased 30 minutes after salt exposure. This increase was the highest expression value for the CRLK1 gene. A decrease can be seen at hour 3 but values increased to baseline levels until the 24-hour period was reached.

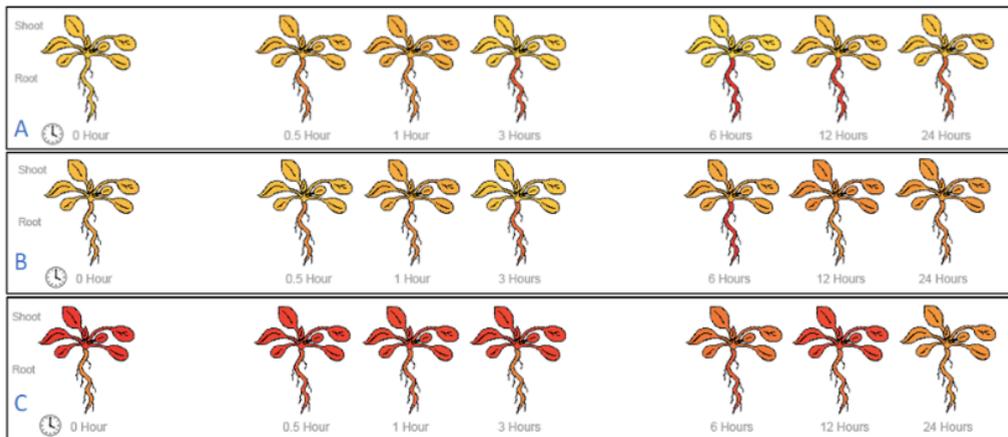


Figure 6. Visualization of gene expression levels of (A) MEKK1, (B) MKK2, and (C) CRLK1 in *Arabidopsis thaliana* after treatment of 150 mM salt (NaCl). Red colouration is indicative of high levels of gene expression while bright yellow represents absence of gene expression. Images were obtained from the *Arabidopsis* eFP browser of the BAR database.

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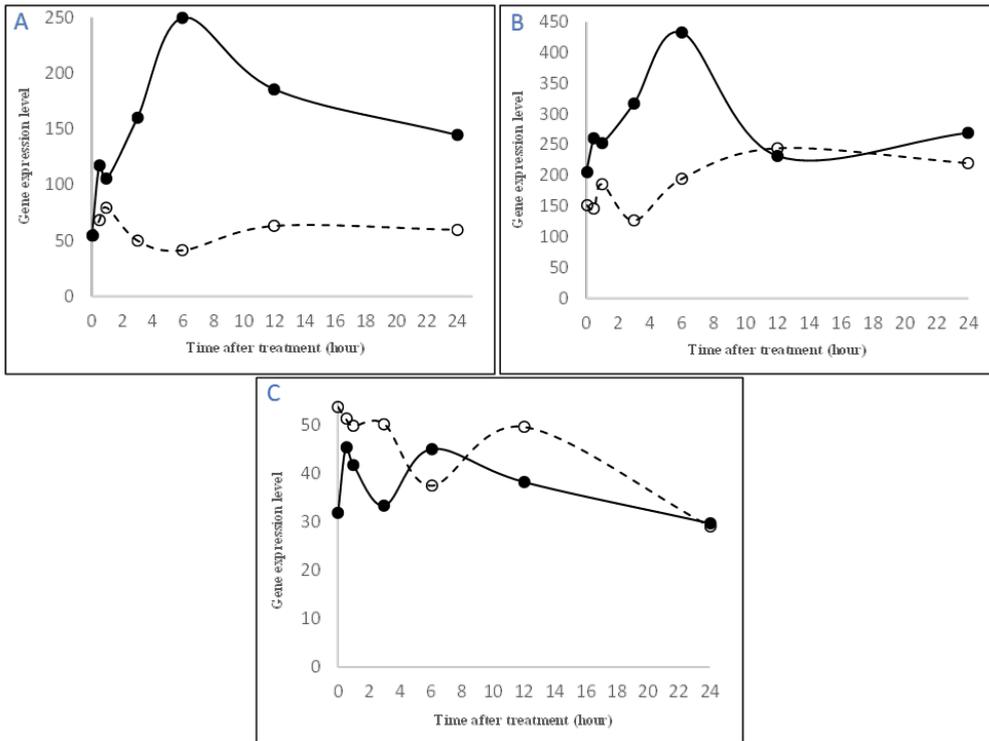


Figure 7. Gene expression pattern of (A) MEKK1, (B) MKK2, and (C) CRLK1 in *Arabidopsis thaliana* after treatment of 150 mM salt (NaCl).

Discussion

Mitogen-activated protein kinase cascades immensely contribute to a plant’s defense system against stresses, predominantly the environmental ones. For instance, salt stress has been known to affect productivity and growth in plants as it induces a subgroup of strains such as ionic, osmotic, and oxidative stresses [YANG & YUO, 2018]. Recent investigations unfolded an increase in soil salinity as a result of aggravating industrial pollution, excessive fertilizer use, and faulty irrigation practices [YANG & YUO, 2018]. Cellular and molecular mechanisms of plants’ defense systems against biotic and abiotic stresses have been one of the major investigative subjects. In this study, we elucidated upstream protein partners of AtMKK1 to advance the understanding on the limited insight concerning salt signaling pathway in *Arabidopsis*. Data mining through bioinformatic tools like STRING and BAR aided in the investigation of interactors. Starting with STRING, network visualization of protein-protein interactions revealed 11 proteins. Among these, AtMEKK1 was identified as an upstream regulator and AtMKK2 established relevance as a redundant protein to AtMKK1. Further data mining through AlphaFold and Uniprot also unfolded another protein partner, CRLK1. Localization of these target genes through BAR displayed expression in many organ, tissue, and subcellular locations. Despite varying intensity in gene expressions across different locations, the observation that these genes are in fact vastly distributed in many plant tissues secures the

expectation that they are able to transduce signals more productively. In addition, knowing their presence in extensive tissue locations also suggests that these genes can be expressed into proteins for the purpose of inducing various biological processes. On the other hand, co-expression of the target genes revealed that their expression levels after salt treatment predominantly increased. This observation again supports the notion that they play biological roles in the defense signaling system against salinity.

Downstream MAPK cascades are heavily dependent on phosphorylation of proteins to amplify specific stimuli and elicit a physiological and biochemical response. Understanding protein functions and the mechanism behind their activation are imperative in this built-in system. A previous study has established that AtMEKK1 expression enabled prolonged survival of yeast under intense salinity as a result of increased glycerol synthesis [COVIC & al. 1999]. The researchers also observed that among cold, water, and salt stresses, highest levels of AtMEKK1 expression was induced by salt stress [COVIC & al. 1999]. More importantly, AtMEKK1 phosphorylated downstream AtMKK2 [FURUYA & al. 2013]. Molecular evidences suggested that AtMKK2 shares similar and specific functions with AtMKK1 [QIU & al. 2008]. Among other molecular roles, both proteins interact with downstream MPK4 which is a protein associated with the jasmonate (JA) signaling pathway [TEIGE & al. 2004]. The JA pathway has been found to inhibit cell division and elongation in the root tissues in order to steer clear of high salt concentration in the soil [VALENZUELA & al. 2016]. Finally, CRLK1 has been demonstrated to interact with MEKK1 in the regulation of MAPK cascades during cold stress [YANG & al. 2010]. This establishes relevance in this study as CRLK1 could potentially be implicated in the salt stress signaling pathway as well, given the overlap in the activation cascade following MEKK1 phosphorylation between cold stress and salinity.

Dependence on plants as sustenance is one of the reasons behind growing scientific investigations that aim to better plant production and resistance against stresses. Today, research on creating genetically engineered plants is increasing. Advancing our understanding on molecular mechanisms behind the elaborated plant systems that enable defense and resistance can potentially lead us closer to filling the gap and using findings to genetically modify plant species, especially those that are more prone to stresses.

Acknowledgements

This work was supported by a research grant to Tim XING from Bayer Crop Science Inc. and Carleton University, Canada.

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How to cite this article:

ARMAS F. & XING T. 2022. Investigating protein partners of AtMKK1 as part of the MAPK signaling pathway during salt stress. *J. Plant Develop.* **29**: 141-150. <https://doi.org/10.47743/jpd.2022.29.1.917>
