University of California San Diego

Chemical-Genetic Characterization of *VICTR* & *RDA2* Homologous Genes in the Regulation of ABA and DFPM Signal-Transduction in Arabidopsis Plants

A thesis submitted in partial satisfaction of the requirements for the degree Master of Science

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Chemistry

by

Eduardo Ramirez

Committee in charge:

Julian I Schroeder, Chair
Elizabeth A Komives, Co-Chair
Thomas J Bussey
Jin Zhang

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The thesis of Eduardo Ramirez is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-Chair

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Chair

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Abstract of Thesis

Chemical-Genetic Characterization of *VICTR* & *RDA2* Homologous Genes in the Regulation of ABA and DFPM Signal-Transduction in Arabidopsis Plants

by

Eduardo Ramirez

Master of Science in Chemistry

University of California San Diego, 2019

Professor Julian I. Schroeder, Chair
Professor Elizabeth A. Komives, Co-Chair

The hormone abscisic acid (ABA) triggers signal transduction that mediates drought resistance. Through a chemical genetics approach, a synthetic small molecule "DFPM" ([5-(3,4-dichlorophenyl) furan-2-yl]-piperidine-1- ylmethanethione) was shown to stimulate plant defense related genes and negatively regulates ABA signaling. DFPM independently induces root growth arrest in *Arabidopsis thaliana* through the *VARIATION IN COMPOUND TRIGGERRED ROOT growth response* (*VICTR*). *VICTR* encodes for Toll-Interleukin 1 Receptor-nucleotide binding

Leucine-rich repeat (TIR-NB-NLR) protein. DFPM-screening has led to the identification of the ***R****ESISTANCE TO* ***D****FPM-INHIBITION OF* ***A****BA SIGNALING2 (RDA2)* locus, which encodes for a *Lectin-Receptor Kinase (LecRK). RDA2* plays a key role in DFPM-mediated inhibition of ABA signal-transduction and phosphorylating mitogen-activated protein kinases (MAPKs) 3 & 6 (MPK3MPK6). Two genes that lie upstream of RDA2 were characterized as *LecRKs* and found to play a role in mannitol-induced stressed, thus naming them ***E****NHANCED SHOOT* ***G****ROWTH UNDER* ***M****ANNITOL STRESS (EGM) 1 & 2 (EGM1 & EGM2).* **I am interested in investigating the role of VICTR and its homologous genes regulating ABA signaling-transduction after exposure to DFPM. This project also aims to understand the role of the *EGM* homologous genes with respect to RDA2 after exposure to DFPM.** In order to study the role of *VICTR* and its homologous tandem genes I utilized Near Isogenic Lines (NILs) NIL-Col-0 which contains functional *VICTR* homologs, and NIL-Bu-5 which does not contain any *VICTR* homologs. I also used mutant lines of *RDA2* (*rda2-1* and *rda2-2*) as well as T-DNA lines of *EGM1 & 2 (egm1 & egm2)* and an artificial microRNA (amiRNA) line targeting both *EGM* genes *(egm-amiRNA)* to investigate the role of these genes in DFPM-signaling. I first grew seedlings of Col-0, NIL-Col-0, and NIL-Bu-5 for two weeks and treated the plants with various chemical treatments followed by RNA extraction, cDNA synthesis, and qRT-PCR to analyze expression of ABA reporter genes. Using lines of the same genotypes transformed with *pRAB18::GFP* I attempted to compare relative fluorescence after exposure to ABA and DFPM chemical treatments. I also compared DFPM-mediated MAPK-activation across various time points for Col-0 and NILs as well as the *rda2, egm1 & 2,* and *egm-amiRNA* lines. I found that the genetic region comprised *VICTR* and its homologous genes do not play a role in the activation of MAP Kinase in response to DFPM and are not involved in the crosstalk between ABA and DFPM-signaling transduction.

RDA2 is involved in activation of MAPKs and both *EGM1 and EGM2* are possibly involved in co-regulating *RDA2*. Further analysis and higher order mutants of *VICTR* and *RDA2* homologous genes will allow us to understand their function in ABA and DFPM signaling-transduction.

I. Introduction

1.1 Abscisic Acid and DFPM - signaling pathways

Plants are sessile organisms that continuously need to adapt to their environment in order to survive. Over time plants have evolved sophisticated mechanisms of stress tolerance to cope with environmental changes. An example of a coping mechanism is the regulation of stomata, tiny pores on plant leaves that allow for gas exchange and water loss (Bailey 2019). With the dramatic changes brought upon by global climate change plants are placed in a difficult position where they need to acclimate quickly in the face of new environmental pressures. Examples of such phenomena include drought and pathogen infections which could have detrimental effects on plant growth and crop yields (Wang et al., 2003; Bruce, 2010; Maxmen, 2013; Hatfield and Walthall, 2015). When exposed to both environmental stresses at the same time plant resistance often becomes more reduced (Suzuki et al., 2014; Deutsch et al., 2018), and the two stress-signaling pathways often antagonize each other (MauchMani and Mauch, 2005; Prasch and Sonnewald, 2013; Kim et al., 2011). Because of this, it is important to investigate the mechanisms of stress-tolerance signaling under multiple stresses in order to one day be able to generate plants and crops that can be more adaptive to the changing climate.

The phytohormone abscisic acid (ABA) is a major stress hormone that regulates plant responses to abiotic stresses including drought (Cutler et al., 2010; Finkelstein, 2013; Hauser et al., 2017). ABA responds to biotic and abiotic stress to regulate seed germination, seedling growth and stomatal closure in response to drought (Kim et al., 2012; Finkelstein et al., 2013). When activated ABA first binds to the *Pyrabactin Resistance1 (PYR1*) and *PYR1-like* (*PYL*) receptors which serve as *Regulatory Components of ABA Receptors* (*RCAR*), which in turn respond by inhibiting a clade of protein phosphatases 2C (PP2Cs) (Ma-Lauer et al., 2009; Park et al., 2009; Gonzalez-Guzman et al., 2012). PP2Cs act as regulators for various signaling

pathways (Rodriguez. 1998) such as sucrose nonfermenting 1-related subfamily2 (SnRK2) protein kinases and Ca2+ signaling (Hubbard et al., 2010; Gonzalez-Guzman et al., 2012; Brandt et al., 2015). SnRK2s are released after inhibition of PP2Cs which leads to activation of drought responses such as stomatal closure and expression of ABA reporter genes (Figure 1).

In order to elucidate the interaction between ABA and plant immune signaling a chemical genetic approach was utilized to address genetic redundancy and network robustness in both signaling pathways (Schreiber, 2000; Armstrong et al., 2004; Zouhar et al., 2004; Park et al., 2009). A 9600-compound chemical library was screened for inhibition of ABA signaling which led to the discovery of a small molecule known as DFPM ([5-(3,4-dichlorophenyl)furan-2-yl]-piperidine-1-ylmethanethione)) (Kim et al., 2011). DFPM was shown to inhibit several ABA responses such as rapid disruption of ABA-induced stomatal closure and ABA activation of guard cell anion channels (Kim et al., 2011).

In addition to its role in repressing ABA-signaling, DFPM has also been shown to stimulate effector-triggered immune signaling and expression of plant defense-related genes (Kim et al., 2011). The defense signaling genes *EDS1* (*Enhanced Disease Susceptibility1*), PAD4 (*Phytoalexin Deficient4*), SGT1b (*Suppressor of G2 allele of skp1*), and RAR1 (*Required for Mla12 Resistance*) encode for resistance (R)-proteins which serve as early components of plant immunity and are required for DFPM-mediated inhibition of ABA signal transduction (Kim et al., 2011). DFPM functions at the early-stages of the plant-immunity pathway because of the importance of the resistance (R) proteins recognizing pathogen molecules and activating downstream disease resistance responses (Glazebrook, 2005; Jones and Dangl, 2006). EDS1 and PAD4 interact together and are required for accumulation of Salicylic acid, a plant-defense signaling molecule, which in turn activates local defense reactions and the induction of systemic resistance against pathogens (Durner et al., 1997; Feys et al., 2005; Reitz et al., 2011).

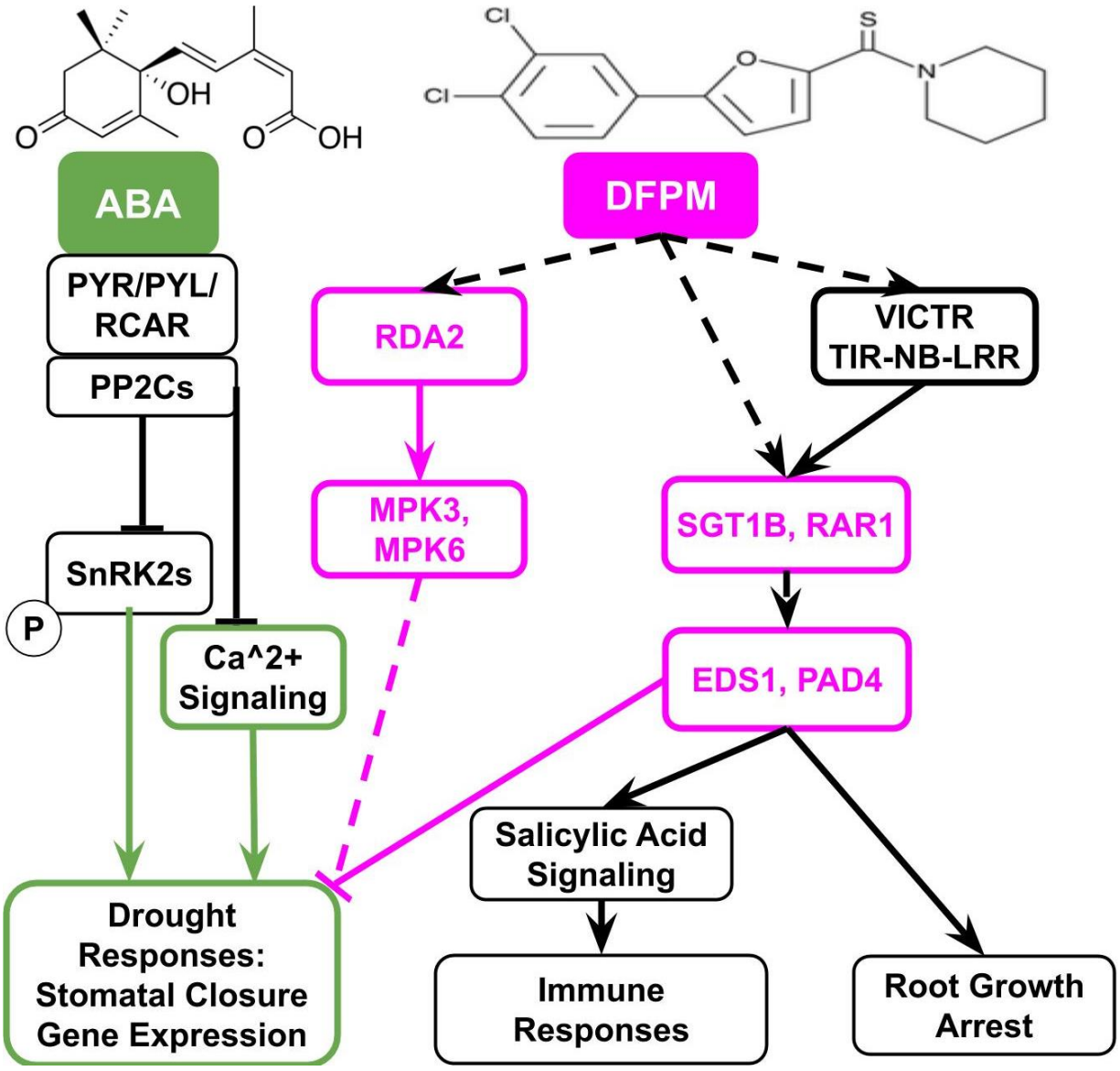


Figure 1. Structure of ABA, DFPM, and proposed pathway illustrating negative regulation of DFPM on ABA signaling and DFPM-medited root growth arrest.
DFPM requires pathogen signaling components such as *EDS1*, *PAD4*, *SGT1B*, *RAR1* near the level of Ca2+ signaling leading to an inhibition of ABA signaling and a repression of drought responses. Early ABA signaling receptors (*PYR*, *PYL* or *RCAR*), *PP2Cs* (type 2C protein phosphatases) and *SnRK2s* (SNF1-related protein kinases) are not affected by DFPM. Downstream signals of the five defense genes such as salicylic acid signaling are not required for DFPM signaling. These same genes are also involved in DFPM-mediated root growth arrest after activation of VICTR. Plant immune signaling components are marked in pink.

1.2 *VICTR-like* genes and Near-Isogenic Lines

After its isolation from a vast chemical screening, the small compound DFPM was shown to be a physiologically small active molecule that inhibits ABA-induced gene expression and signal-transduction (Kim et al., 2011). After screening for additional phenotypic responses, DFPM was shown to cause rapid root growth arrest in Columbia-0 (Col-0) accessions of Arabidopsis Thaliana but not for other ecotypes such as in the Arabidopsis Landsberg erecta (Ler) accession. (Kim et al., 2012). A class of genes known as *VARIATION IN COMPOUND TRIGGERED ROOT growth response* (*VICTR*) was identified from a DFPM-mediated root-growth arrest screening. *VICTR* is a natural genetic variant found in Col-0 ecotypes of Arabidopsis that causes root growth arrest after exposure to DFPM (Kim et al., 2012).

VICTR was further characterized and found to be the gene responsible for DFPM-mediated root growth arrest in Col-0 (Kim et al., 2012). A close homolog of *VICTR* called *VICTR-Like1* (*VICTL*) was characterized and found not to be involved in DFPM-mediated root growth arrest, *VICTR* is the sole gene responsible for this effect (Kim et al., 2012).

VICTR encodes for a *Toll-Interleukin 1 Receptor- nucleotide binding-Leucine-rich repeat* (TIR-NB-NLR) protein that is expressed in root tissue (Kim et al., 2012). TIR-NB-LRR proteins function as immune signaling proteins after activation by R genes in response to pathogens in plants (Belkhadir et al., 2004; Chisholm et al., 2006; Jones and Dangl, 2006; Shen and Schulze-Lefert, 2007; Caplan et al., 2008). *VICTR* was analyzed for its role in Effector-Triggered Immune (ETI) signaling pathway. EDS1 and PAD4 were shown to be required for DFPM-mediated root growth arrest (Kim et al., 2012). In addition, *VICTR* knockout mutants (victr) exhibited low response to DFPM inhibition of ABA-induced stomatal closure signifying that *VICTR* functions upstream of EDS1/PAD4 and is involved in DFPM-induced inhibition of ABA signal transduction (Kim et al., 2012; Figure 1).

After its initial discovery it was unclear whether VICTR acted as a canonical pathogen Resistance (R)-gene requiring a functional nucleotide binding domain (Kim et al., 2012). Because of this new victr alleles exhibiting three independent point mutations in the VICTR gene which lack a nucleotide binding domain were isolated through chemical genetic screening, confirming the role of VICTR as a canonical R-protein functioning in the roots during DFPM signaling due to its role as a TIR-NB-LRR protein (Kunz et al., 2016).

In addition to its role in DFPM-mediated root growth arrest, VICTR also functions as a gene conferring Acquired Osmotolerance which allows plants to withstand stress following initial exposure, giving it the name ACQOS (Ariga et al., 2017). The VICTR/ACQOS locus is found in naturally in Col-0, but not in Bu-5 ecotypes of Arabidopsis Thaliana (Ariga et al., 2017). Two near-isogenic lines (NILs), NIL-Col-0 and NIL-Bu-5, were created by backcrossing the Bu-5 ecotype into Col-0 five times, carrying small chromosomal segments such as VICTR/ACQOS into the background of Col-0 (Ariga et al., 2017). Using these NILs the VICTR/ACQOS locus was narrowed down to a region of 67 kbp containing four TIR-NB-LRR genes (VICTR/ACQOS, VICTL1, and two unidentified NLR genes) in NIL-Col-0 and an unrelated TIR-NB-LRR gene in NIL-Bu-5 (Figure 2; Ariga et al., 2017)**.** My research objective will focus on utilizing both NILs along with wildtype Col-0 to determine whether VICTR and its three tandem-repeat homologs play a role in crosstalk of DFPM interference of ABA signaling.

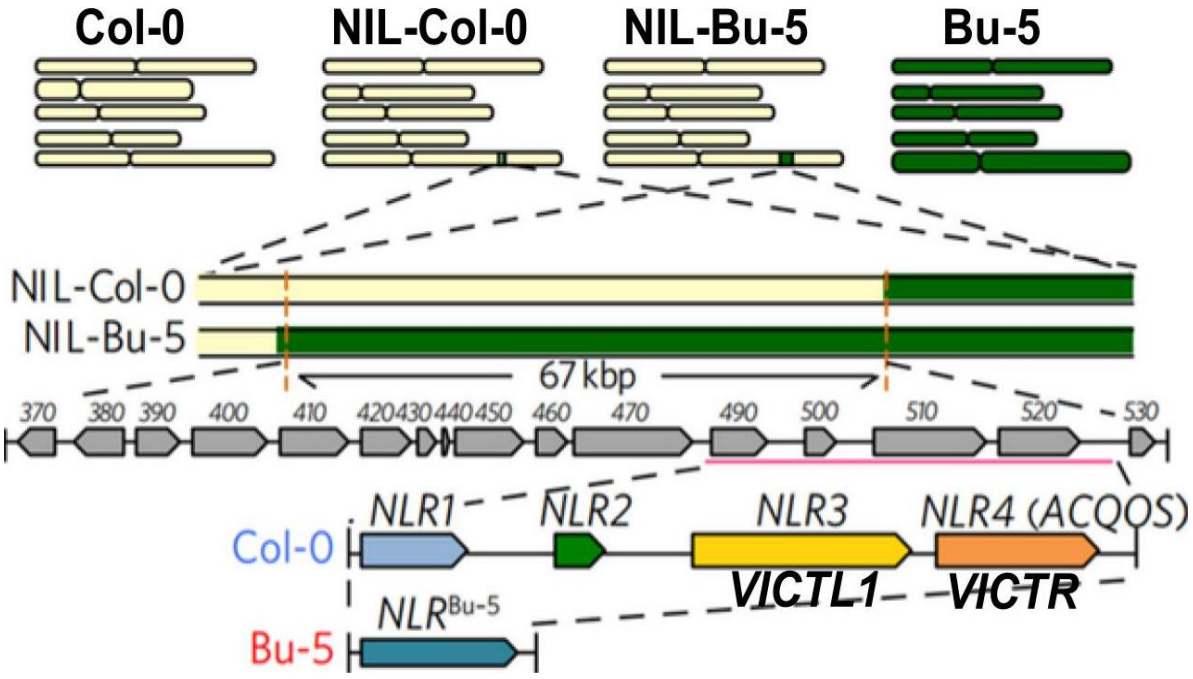


Figure 2. Graphical genotypic representations of Col-0, NIL-Col-0, and NIL-Bu-5 (Data from Figure 1b; Ariga, et al. 2017).
Chromosomal segments of Col-0 are shown in light-yellow and Bu-5 are shown in green. Numbers above the genes are the last three digits of their *Arabidopsis* Genome Initiative (AGI) numbers (At5g46xxx). Two near-isogenic lines, NIL-Col-0 and NIL-Bu-5, were generated by backcrossing *A. thaliana* accession Bu-5 with Col-0 five times. NIL-Col-0 contains a tandem repeat of four *Toll and interleukin1 receptor-nucleotide binding leucine rich repeat* (*TIR-NB-LRR*) genes: *VICTR* (*NLR4*/*ACQS*), *VICTL1* (*NLR3*), and two tandem homologous TIR-NB-LRR genes (*NLR1* & *NLR2*) within a 67 kbp region. NIL-Bu-5 contains one *TIR-NB-LRR* gene (NLR^Bu-5) within the same region originating from the Bu-5 ecotype. Near-isogenic lines serve as a powerful tool for characterizing specific loci.

1.3 Homologous genes of *RDA2*: *EGM1* & *EGM2*

Chemical genetics is a powerful technique that can be used to investigate signaling pathways such as ABA and DFPM-signaling-transduction by circumventing genetic redundancy, mutant lethality, and network robustness (Park et al., 2009; Toth and Van Der Hoorn, 2010; Park et al., 2019). The gene ***R****ESISTANCE TO* ***D****FPM-INHIBITION OF* ***A****BA SIGNALING****2*** *(RDA2)* was recently identified through a chemical genetics screen and was found to play a key role in DFPM-mediated inhibition of ABA signal-transduction (Park et al., 2019). *RDA2* is required for DFPM-mediated activation of immune signaling components such as phosphorylation of mitogen-activated protein kinases (MAPK) 3 and 6 (MPK3/MPK6) (Park et al., 2019).

Two genes that lay upstream of *RDA2* were identified through a chemical genetics screen and found to confer seedling growth under mannitol exposure (a known substitute for drought stress assays (Verslues et al., 2006)) thus naming them ***E****NHANCED SHOOT* ***G****ROWTH UNDER* ***M****ANNITOL STRESS (EGM) 1 & 2 (EGM1/EGM2)* (Trontin et al., 2014). *EGM1* and *EGM2* are thought to be involved in biotic stress responses mediated by exposure to mannitol (Trontin et al., 2014). *EGM1 & EGM2* could function in plant defense response due to mannitol being a fungal pathogen secretor (Velez et al., 2008) and not known to accumulate naturally in plants (Stoop et al., 1996; Klepek et al., 2005; Reinders et al., 2005; Trontin et al., 2014).

RDA2 along with its tandem homologs *EGM1 & EGM2*, encode for putative *Lectin Receptor Kinases* (*LecRKs*; Park et al., 2019; Trontin et al., 2014). *LecRKs* are involved in immune signaling by perceiving pathogen-associated molecular pattern signals (PAMP) during pathogen infection and activate PAMP-triggered immune (PTI) responses to combat infection (Chrispeels and Raikhel, 1991; Chen et al., 2006, 2017; Singh and Zimmerli, 2013; Vaid et al., 2013; Lannoo and Van Damme, 2014; Ranf et al., 2015; Bellande et al., 2017; Wang et al., 2017). *LecRKs* classify as Receptor-Like Kinases (RLK) and are involved in plant responses such as development, growth, hormone perception, and pathogen response (Goff et al., 2007).

DFPM works by stimulating effector-triggered immune signaling and expresses plant defense-related genes in plants (Kim et al., 2011). The focus of my second research project is to investigate the role of *RDA2* and its two closest tandem homologs *EGM1* and *EGM2* in the plant-immune signaling pathway using DFPM as a medium to instigate pathogen-signaling. I will specifically address how these three tandem homologous genes are involved in the activation of mitogen-activated protein kinases (MAPKs), which acts as an early cellular PTI-response (Meng and Zhang, 2013; Frei Dit Frey et al., 2014), after exposure to DFPM and identify potential connections between these three homologous genes.

II. Experimental Methods

2.1 Plant Materials & Growing Conditions

Arabidopsis Thaliana accession Columbia-0 (Col-0) was primarily used throughout this study. Seeds of the Near-Isogenic Lines NIL-Col-0 and NIL-Bu-5 were provided by Professor Teruaki Taji of Ariga et al., 2017. Mutant lines of *RDA2*: *rda2-1* and *rda2-2* were generated via ethyl-methanesulfonate (EMS) mutagenesis and T-DNA insertion respectively (Park et al., 2019). Single mutants of the EGM genes: *egm1* and *egm2* were made from T-DNA insertions and an artificial- microRNA (amiRNA) line that targets both EGM genes (named *egm-amiRNA*) were provided by Professor Olivier Loudet Trontin et al., 2014. All lines used in this study were generated in the background of the Col-0 ecotype. Arabidopsis seeds were sterilized with 10% bleach for 15 minutes, then rinsed seven times with sterile water and sown onto ½ Murashige and Skoog (MS) media (pH 5.8) plates with 1% sucrose (MSS) and 0.8% Phyto agar. Plates were then kept in the dark for 2-3 days at 4°C and transferred to a growth chamber under a 16/8 hour light & dark cycle at 60-80 umol m-2 sec-1light intensity at 19-21°C.

2.2 Genomic DNA Extractions and Genotyping Analysis

In order to verify the validity of the Arabidopsis lines, genomic DNA extractions and subsequent genotyping using polymerase-chain reaction (PCR) and gel electrophoresis was utilized. After 1-2 weeks of growth on ½ MSS media, plants were transferred to soil and grown for an additional 1-2 weeks before collecting leaf tissue for genomic DNA extraction. Leaf tissue was then frozen in liquid nitrogen and ground using a Retsch MM301 mixer mill. The grounded tissue was then treated with genomic DNA extraction buffer containing 50 mM Tris-Cl, 10 mM EDTA, 100 mM NaCl, 1% SDS, and 10 mM β-mercaptoethanol, incubated for 10 min at 65°C and treated with potassium acetate at 1/3 volume on ice. The mixture was centrifuged at 10,000g at 4°C. Following centrifugation, the supernatant was removed and the

genomic DNA was precipitated with isopropanol and centrifuged again at 10,000g at 4°C. Isopropanol was removed and the pelleted DNA washed with 80% ethanol before being dried and dissolved in water. Afterwards genomic DNA was then used in a mixture containing water, 10X Taq buffer with KCl and 15 mM MgCl2, 10 mM deoxynucleoside triphosphate (dNTPs), 10 mM of Forward and Reverse Primer specific to each gene (Table 1), and Taq Polymerase; the mixture was then taken to a PCR to amplify genes of interest; melting temperature 95°C for 40 sec, annealing temperature 58°C for 30 sec, elongated temperature 72°C for 1 min, and repeated 40 times. Finally, the amplified DNA bands were visualized using Gel Electrophoresis by running on a 1% Agarose gel at 80 volts for 15-20 minutes. Using this technique, I was able to confirm the validity and genotype of all the lines used in this study.

2.3 Quantitative Gene Expression Analysis

After growing in the growth chamber for 14 days seedlings were transferred and incubated in ½ MSS liquid media. Plants were pretreated with 30 μM DFPM or solvent control (0.06% DMSO) for 1 hour, then incubated in 10 μM ABA or solvent control (0.02% Ethanol) for 5 hours. This was followed by RNA extraction, plant tissue was frozen in liquid nitrogen and grounded using a Retsch MM301 mixer mill. Total RNA was extracted using SpectrumTM Plant Total RNA Kit (Sigma-Aldrich, St. Louis, MO, US). Quantification of RNA-extracts was performed via Nanodrop and subsequent cleaning using TURBOTM DNase (Thermo-Fisher Scientific, Vilnius, Lithuania). Complementary-DNA was reverse-transcribed using First-Strand cDNA Synthesis Kit (GE Healthcare, Little Chalfont, UK). Quantitative Reverse Transcriptase-PCR (qRT-PCR) was used to analyze expression of the ABA reporters *RAB18, RD29A, ERD10* and housekeeping-gene *PDF2*. qRT-PCR was performed on a BioRad CFX96 qPCR System (BioRad, Hercules, CA, US) using SYBR Green JumpStart (Sigma-Aldrich, St. Louis, MO, US) with gene-specific primers (Table 1). Levels of transcript were normalized against the

housekeeping gene PDF2. Statistical analyses were performed using Microsoft Excel.

2.4 Measurement of phosphorylated mitogen-activated protein kinases

14 day-old seedlings were transferred and incubated in ½ MS liquid media with 1% sucrose. Plants were incubated in 30 μM DFPM for the specified time points (15, 30, and 60 minutes), solvent control (0.06% DMSO) was used for the 0 minute time point. This was followed by protein extraction; total proteins were extracted in extraction buffer [25 mM Tris-HCl (pH 7.8), 75 mM NaCl, 10 mM MgCl2, 15 mM EGTA, 1 mM dithiothreitol (DTT), 1 mM NaF, 0.5 mM NaVO3, 15 mM b-glycerolphosphate, 15 mM p-nitrophenylphosphate, 0.1% Tween 20, 0.5 mM phenylmethylsulfonyl fluoride, 5 lg ml1 leupeptin]. The concentration of protein extract was measured using Bradford protein assay (He 2011). Twenty micrograms of total proteins for each genotype and treatment was subjected to SDS– polyacrylamide gel electrophoresis (PAGE). Following this, protein samples were transferred from SDS-PAGE gel to Polyvinylidene fluoride (PVDF) membrane in a sandwich of Whatman paper, PVDF membrane, SDS gel, and Whatman paper; and ran ~100 mA (Park 2007). After blocking PVDF membrane in 5% Tris-buffered Saline with 1% Tween-20, Western blotting was performed using antibodies specific for MPK3 & MPK6, anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/ Tyr204) monoclonal antibody (9101, Cell Signaling Technology, Danvers, MA, US) and the secondary anti-rabbit antibody (BioRad, Hercules, CA, US) by the manufacturer's protocol. Blots were stained with Coomassie blue solution to visualize the amount of loaded proteins.

2.5 *pRAB18::GFP* agrobacterium transformation and Microscopy Analysis

In order to obtain lines of Col-0, NIL-Col-0, NIL-Bu-5, and victr expressing pRAB18::GFP each line was transformed with agrobacterium via floral dipping as described in *Agrobacterium*-mediated transformation of *Arabidopsis thaliana* using the floral dip method by Zhang et al., 2006. Seedlings were then screened in the T1 generation using kanamycin and

cefotaxime. In the T2 generation seeds were separated by comparing GFP fluorescence using a GFP-dissecting microscope. Arabidopsis seeds expressing *pRAB18::GFP* were germinated on ½ MSS with 0.8% Phyto agar, and grown in the growth chamber at 60–80 μmol m−2 sec−1 light intensity at 19–21°C . After 7-days plants were transferred to soil and grown under a 12/12 h light/dark cycle growth chamber at 60–80 μmol m−2 sec−1 light intensity at 19–21°C for 7 more days. First and second true leaves were removed and incubated on ½ MS liquid medium with 1% sucrose. Each leaf was placed on a well of a 24-well plate with the abaxial (bottom) leaf surface exposed to the medium. DFPM (or its respective control DMSO) was added to a final concentration of 10 μm (0.02% DMSO). After an hour of incubation ABA (or its respective control Ethanol) was added to a final concentration of 20 μm (0.04% EtOH). GFP fluorescence signals were observed from abaxial epidermal layers of leaves using confocal microscopy, after 24 hours of ABA addition. Average fluorescence intensity of each image was calculated using Image J software in a genotype and chemical treatment-blind manner. Statistical analyses were performed using Microsoft Excel.

Table 1. List of Primers for Genotyping and qRT-PCR Analysis

NAME	SEQUENCE	Purpose	DESCRIPTION
At5g_126-F	**5'- AAC CGA CGA ATA AAG AAG ATG A -3'**	**Genotyping NILs**	**Targets region to the left of 67kbp; NIL-Col-0 and NIL-Bu-5 have DNA from Col-0**
At5g_126-R	**5'- AAC GAC AAC TCG CCG AAC -3'**	**Genotyping NILs**	**Targets region to the left of 67kbp; NIL-Col-0 and NIL-Bu-5 have DNA from Col-0**
At5g_128-F	**5'- GAC TCC ATC CGC CAA TAA AT -3'**	**Genotyping NILs**	**Targets region of 67kbp; NIL-Col-0 and NIL-Bu-5 contain different DNA**

Table 1 Continued. List of Primers for Genotyping and qRT-PCR Analysis

At5g_128-R	**5'- CCA TTG TTT CTC AGA CTT TTC C -3'**	**Genotyping NILs**	**Targets region of 67kbp; NIL-Col-0 and NIL-Bu-5 contain different DNA**
At5g_136-F	**5'- GAG TTG TGG ATT CTA GAC GAT CT -3'**	**Genotyping NILs**	**Targets region to the right of 67kbp; NIL-Col-0 and NIL-Bu-5 have DNA from Bu-5**
At5g_136-R	**5'- GTT CCA CGA GAG TCC AAA TGT T -3'**	**Genotyping NILs**	**Targets region to the right of 67kbp; NIL-Col-0 and NIL-Bu-5 have DNA from Bu-5**
RAB18-F	**5'- ACA TGG CGT CTT ACC AGA ACC G -3'**	**qRT-PCR of ABA reporter genes**	**Amplify expression of RAB18 reporter**
RAB18-R	**5'- TAC TGC TGC TGG ATC GGG TTC -3'**	**qRT-PCR of ABA reporter genes**	**Amplify expression of RAB18 reporter**
RD29A-F	**5'- ATC ACT TGG CTC CAC TGT TGT TC -3'**	**qRT-PCR of ABA reporter genes**	**Amplify expression of RD29A reporter**
RD29A-R	**5'- ACA AAA CAC ACA TAA ACA TCC AAA GT -3'**	**qRT-PCR of ABA reporter genes**	**Amplify expression of RD29A reporter**
ERD10-F	**5'- TCT CGC GTC TGA GTT TGA GCA C -3'**	**qRT-PCR of ABA reporter genes**	**Amplify expression of ERD10 reporter**
PDF2-F	**5'- TAA CGT GGC CAA AAT GAT GC -3'**	**Normalizing qRT-PCR of ABA reporter genes**	**Amplify expression of PDF2 housekeeping gene**
PDF2-R	**5-' GTT CTC CAC AAC CGC TTG GT -3'**	**Normalizing qRT-PCR of ABA reporter genes**	**Amplify expression of PDF2 housekeeping gene**
EGM1-F	**5'- AAG AAG TTT TGC CCT CCT TGA G -3'**	**Genotyping egm T-DNA mutants**	**Targets functional EGM1 gene**
EGM1-R	**5'- GAT CGG TAC AAT ATT GGC AGG C -3'**	**Genotyping egm T-DNA mutants**	**Targets EGM1 gene tail-end**
LBb1.3	**5'- ATT TTG CCG ATT TCG GAA C -3'**	**Genotyping egm T-DNA mutants**	**Targets T-DNA inserted in EGM1**

Table 1 Continued. List of Primers for Genotyping and qRT-PCR Analysis

EGM2-F	**5'- AAG AAG TTT TGC TTC CCT TGG -3'**	**Genotyping egm T-DNA mutants**	**Targets functional EGM2 gene**
EGM2-R	**5'- CTG ACT TTG CAA GAA GAT CCG -3'**	**Genotyping egm T-DNA mutants**	**Targets EGM2 gene tail-end**
WISC LB-P745	**5'- AAC GTC CGC AAT GTG TTA TTA AGT TGT C -3'**	**Genotyping egm T-DNA mutants**	**Targets T-DNA inserted in EGM2**
EGM-amiRNA-F	**5'- ACG CTC GGA CGC ATA TTA CA -3'**	**Genotyping egm amiRNA line**	**Targets amiRNA located in EGM genes**
EGM-amiRNA-F	**5'- GAG AGA CCC GTA CAA ACT GTG A -3'**	**Genotyping egm amiRNA line**	**Targets amiRNA located in EGM genes**

III. Results

3.1 ABA-induced Gene Expression Analysis of Near-Isogenic Lines

It remains unknown whether the *VICTR TIR-NB-LRR* receptor or its close homologs affect DFPM-inhibition of ABA signaling. In order to investigate the role of *VICTR* and *VICTR-like* genes with respect to the ABA and DFPM-signaling pathways a quantitative reverse-transcribed-polymerase chain reaction (qRT-PCR) was performed. Gene expression was examined in 14-day-old plants of Col-0, NIL-Col-0, and NIL-Bu-5 using ABA-reporter genes after exposure to DFPM, ABA, or respective controls (0.06% DMSO, 0.02% ethanol).

The genes *RESPONSIVE TO ABA 18* (*RAB18*), *RESPONSIVE TO DESICCATION 29A* (*RD29A*), and *EARLY RESPONSIVE TO DEHYDRATION 10* (*ERD10*) were utilized as reporters because they are upregulated in the presence of ABA or drought (Wu et al., 2017). To account for variation across ABA-induced gene expression, the housekeeping gene *PROTODERMAL FACTOR 2* (*PDF2*) was used to normalize expression levels of *RAB18*, *RD29A*, and *ERD10* due to its high-level of stability and identity in other plant species (Lilly et al., 2010).

After analysis of qRT-PCR we observe an increase in gene expression under the ABA condition (Figure 3a-c). Gene expression became comparable to the control in DFPM. Under the ABA/DFPM chemical condition there was a noticeable decrease in gene expression when compared to ABA (Figure 3a-c). This trend was observed across the three genotypes. There was no noticeable difference in DFPM-inhibition of ABA-mediated gene expression when comparing gene expression between genotypes. These data suggests that VICTR and VICTR-like genes are not involved in the crosstalk-interaction between ABA and DFPM signaling.

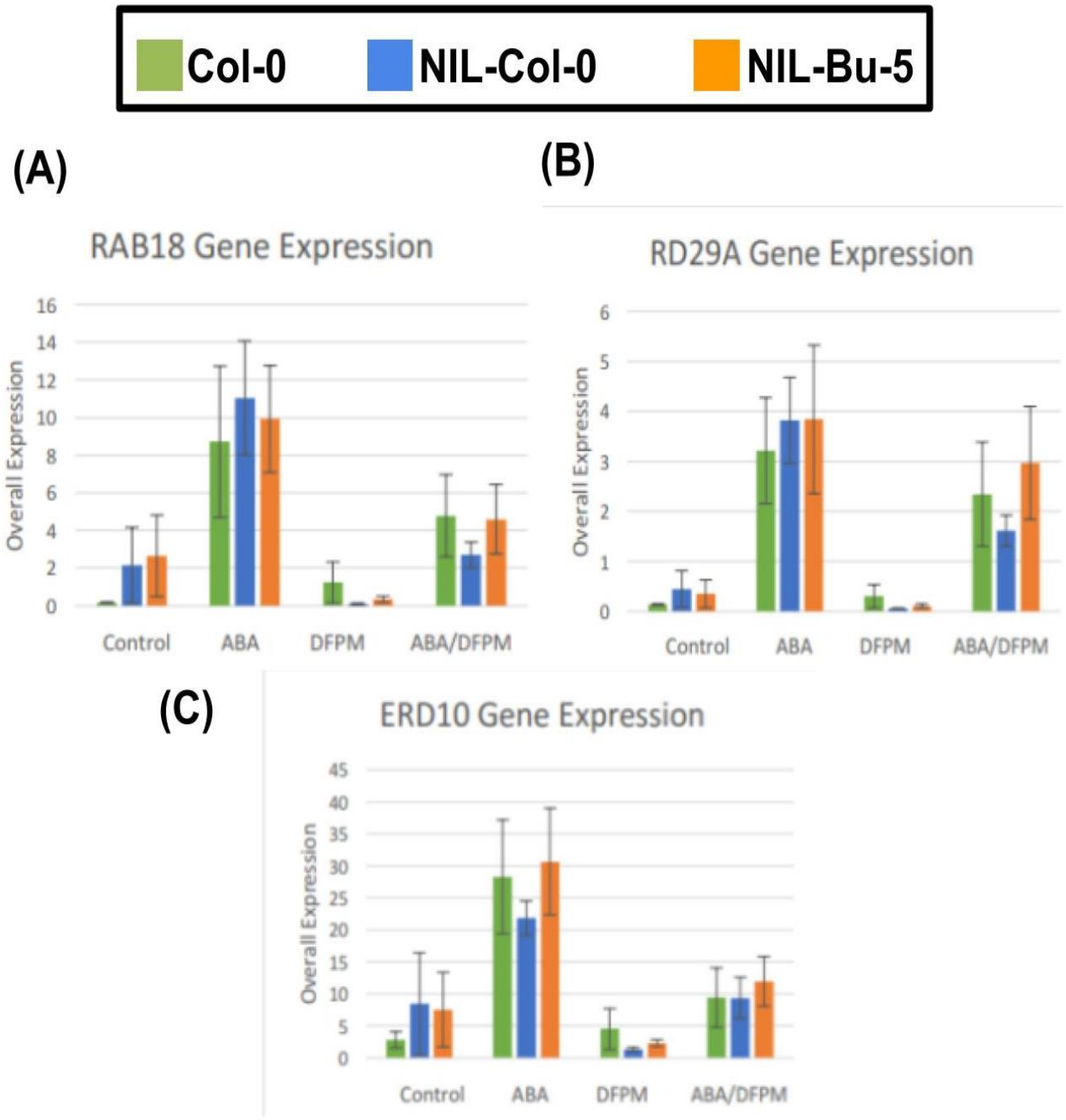


Figure 3a-c. quantitative real-time-polymerase chain reaction (qRT-PCR) analysis of *RAB18, RD29A*,and *ERD10* gene expression.
DFPM-inhibition of ABA-mediated gene expression shows no difference between NIL-Bu-5 compared to the control NIL-Col-0. It appears that VICTR does not affect the interference with ABA signaling. Expression levels of ABA responsive genes were monitored after being pre-treated with 30 µM DFPM or solvent control (0.06% DMSO) for 1 hour followed by 10 µM ABA or solvent control (0.02% ethanol) for 5 hours. Expression levels were normalized with the housekeeping gene *PDF2*. Results are averages of n = 4 biological replicates.

3.2 DFPM-mediated MAPK activation of Near-Isogenic Lines

When plants are exposed to DFPM immune signaling genes including *Pathogenesis related 5* (*PR5*) are activated in an EDS1/PAD4-dependent manner (Kim et al., 2011, 2012). *EDS1* (*Enhanced Disease Susceptibility 1*) and *PAD4* (*Phytoalexin Deficient 4*) are defense-signaling genes required for the accumulation of salicylic acid (Feys et al., 2004) and are core-components in DFPM-mediated effector-triggered immunity (ETI) (Bhattacharjee et al., 2011; Heidrich et al., 2011) including DFPM-mediated inhibition of ABA signal-transduction (Kim et al., 2011, 2012). Because of this we want to compare DFPM-mediated activation of mitogen-activated protein kinases (MAPKs) which are examples of early cellular response in pathogen-associated molecular pattern (PAMP)-triggered immune signaling (PTI) (Meng and Zhang, 2013; Frei Dit Frey et al., 2014). MAPK cascades including MAP kinase kinase 4 (MKK4) and MKK5 are involved in DFPM-mediated interference signaling (Park et al., 2019).

Using NIL-Col-0 and NIL-Bu-5 we investigated whether the absence of *VICTR* and *VICTR-like* genes alters the activation of MAPKs after induction by DFPM. DFPM can activate MAPK-proteins MPK3 and MPK6 in a manner similar to flagellin 22 (flg22), a bacterial flagellin that elicits PTI-signaling via *FLS2* (Park et al., 2019; Asai et al., 2002). After exposing plants of Col-0, NIL-Col-0, and NIL-Bu-5 to DFPM we observe no clear difference in the levels of phosphorylated MPK3 and MPK6 across four biological replicates (Figure 4a-d). The genetic region consisting of *VICTR*, *VICTL1*, and two additional *VICTR* homologous *TIR-NB-LRR* genes do not contribute to the activation of MAPKs after exposure to DFPM (Park et al., 2019).

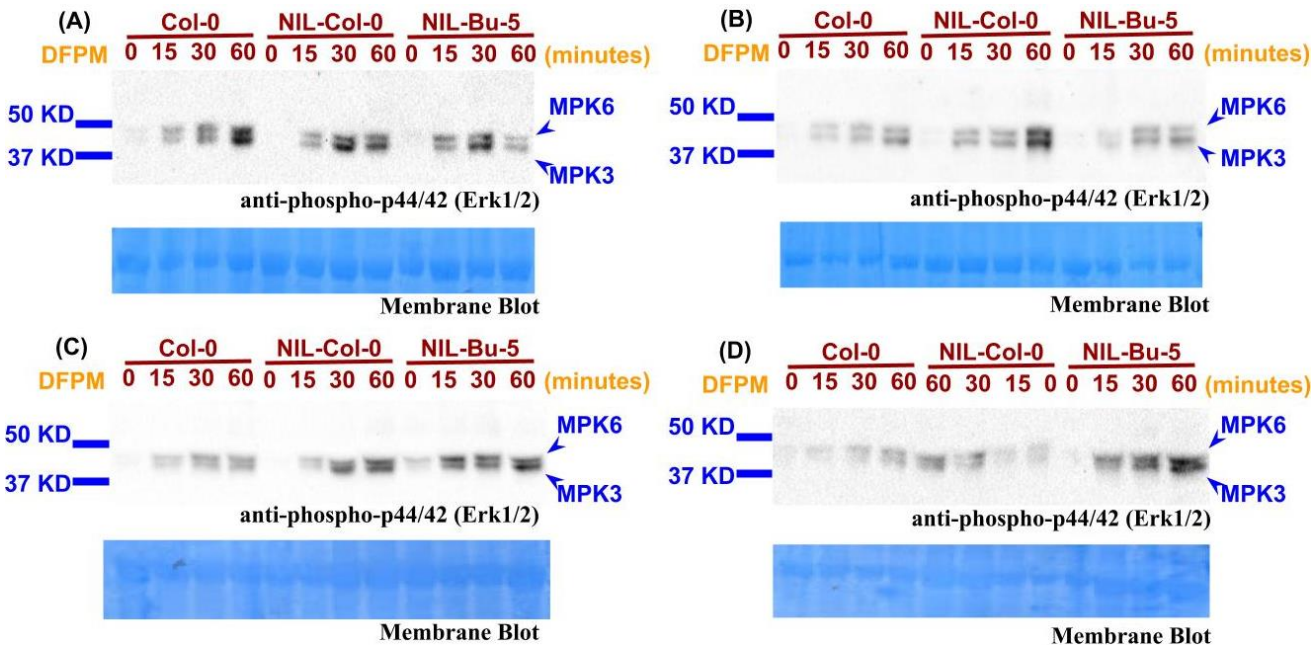


Figure 4a-d. DFPM-mediated activation of mitogen- activated protein kinases (MAPKs) in Col-0, NIL-Col-0, and NIL-Bu-5 (Data from Figure 5d; Park, et al. 2019).
None of the near isogenic lines (NIL-Col-0/NIL-Bu-5) exhibited a clear disruption in DFPM-mediated activation of MAPKs compared to wildtype (Col-0). Plants were treated with 30 µM DFPM for the indicated time points and used for whole protein extraction and subsequent Western blotting. Phosphorylated MAPKs were detected using anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) antibody. Loading controls: Coomassie blue staining of membranes. Results from four independent replicates (a-d) are shown.

3.3 DFPM-mediated MAPK activation of RDA2 mutants

RDA2 encodes a putative lectin receptor kinase (LecRK; Park et al., 2019). LecRKs perceive PAMP signals during pathogen infection and activate PTI-signaling as to combat infection (Chrispeels and Raikhel, 1991; Chen et al., 2006, 2017; Singh and Zimmerli, 2013; Vaid et al., 2013; Lannoo and Van Damme, 2014; Ranf et al., 2015; Bellande et al., 2017; Wang et al., 2017). In addition to activating immune signaling, it was learned that *RDA2* inhibits ABA signal transduction and is required for perceiving a signal derived from DFPM and transduces immune responses including DFPM-mediated activation of MAPKs (Park et al., 2019).

Here we utilized two mutant alleles of *RDA2*, *rda2-1* and *rda2-2* generated via EMS-mutagenesis and T-DNA insertion respectively (Park et al., 2019), and compared their MAPK-activation to wild type Col-0 and Col-0 with *pRAB18::GFP* after exposure to DFPM to determine whether *RDA2* is essential for DFPM-mediated activation of MAPKs. *rda2* mutant plants exposed to DFPM were examined along with wild-type plants. Levels of phosphorylated MPK3 and MPK6 were clearly reduced in the *rda2-1* and *rda2-2* mutants treated with DFPM when compared with wild-type across three biological replicates (Figure 5a-c). This result shows that *RDA2* is an essential gene for DFPM-mediated activation of MAPKs (Park et al., 2019).

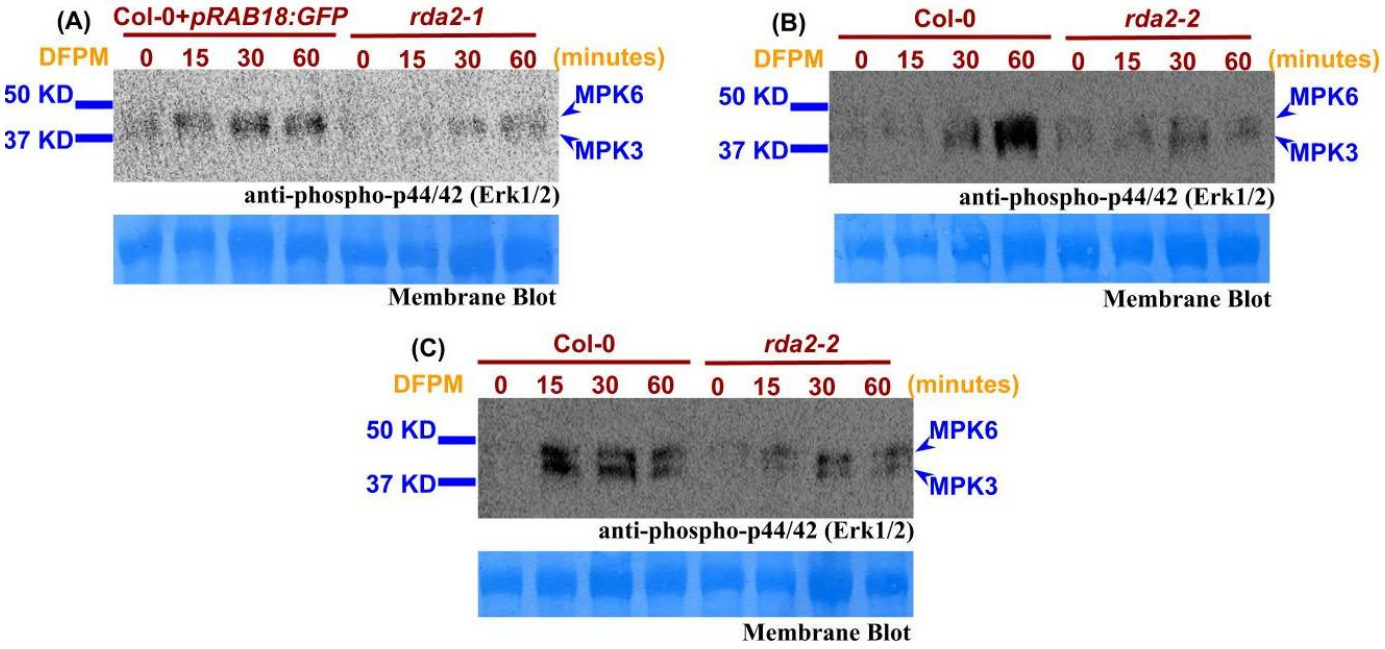


Figure 5a-c. DFPM-mediated activation of mitogen- activated protein kinases (MAPKs) between wild-type and RDA2 mutants (Data from Figure 4c; Park, et al. 2019).
DFPM-mediated activation of MAPKs was diminished in rda2-1 and rda2-2 mutants compared with the corresponding wild-type plants. Col-0 expressing pRAB18::GFP (WT pRAB18::GFP) was used as a wild-type control for rda2-1, and Col-0 (WT) was used as a wild-type control for rda2-2. Plants were treated with 30 µM DFPM for the indicated time points and used for whole protein extraction and subsequent Western blotting. Phosphorylated MAPKs were detected using anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) antibody. Loading controls: Coomassie blue staining of membranes. Results from three independent replicates (a-c) are shown.

3.4 DFPM-mediated MAPK activation of EGM mutants and amiRNA

Two genes that are in tandem to *RDA2 (At1g11330)*, *EGM1 (At1g11300)* and *EGM2 (At1g11303),* also encode for G-type (Galanthus nivalis agglutinin-type) Lectin Receptor Kinase (G-LecRKs) and are involved in plant-shoot growth and signaling of mannitol-associated stress response (Trontin et al., 2014; Bellande et al., 2017; Teixeira et al., 2018). It has been proposed that the receptor-like kinases encoded by *EGM1* and *EGM2* could contribute to plant defense after activation by mannitol produced by pathogens such as fungi (Trontin et al., 2014; Bellande et al., 2017). Because of their close proximity and the fact that they are members of the same gene family as *RDA2,* we wanted to investigate whether *EGM1* and *EGM2* play a role on activating immune-signaling after exposure to DFPM, similar to *RDA2*.

A DFPM-mediated MAPK assay using T-DNA insertion mutants of *EGM1* and *EGM2*, *egm1* and *egm2* respectively (Trontin et al., 2014), was performed. I compared their MAPK activation to wild type Col-0 after exposure to DFPM across different time periods. Although there is variation in the strength of MAPK-activation, the MAPK proteins observed in *egm1* and *egm2* were comparable to wildtype (Figure 6a-d). These findings contrast the measurable impairment in DFPM-mediated activation of MAPKin the *rda2* mutants (Figure 5a-c). Thse data suggests that the individual mutations of *egm1* and *egm2* do not result in a loss of MAPK activity. It appears that *EGM1* and *EGM2* are not necessary for DFPM-mediated MAPK activation. *EGM1* and *EGM2* could serve as complementary genes to each other, a mutation in either gene is complimented by the presence of the other. From the present data it is unclear whether *EGM1* and *EGM2* are associated with DFPM-mediated MAPK-activation of *RDA2*.

In addition to the *egm1* and *egm2* T-DNA insertion mutants, an artifical-microRNA (amiRNA) that targets *EGM1* and *EGM2* was generated by Trontin et al., 2014. AmiRNAs serve as a powerful tool for controlling expression of closely related genes (Eamens et al., 2014). The

amiRNA line targetting *EGM1* and *EGM2* (named egm-amiRNA from here onwards) shows a decrease in relative expression of *EGM1* and *EGM2* under both normal and mannitol-induced conditions (Trontin et al., 2014). Using the egm-amiRNA line we can investigate the roles of *EGM1* and *EGM2* simultaneously in the DFPM-mediated activation of immune responses and explore the relationship between the two *EGM* genes and their connection to *RDA2*.

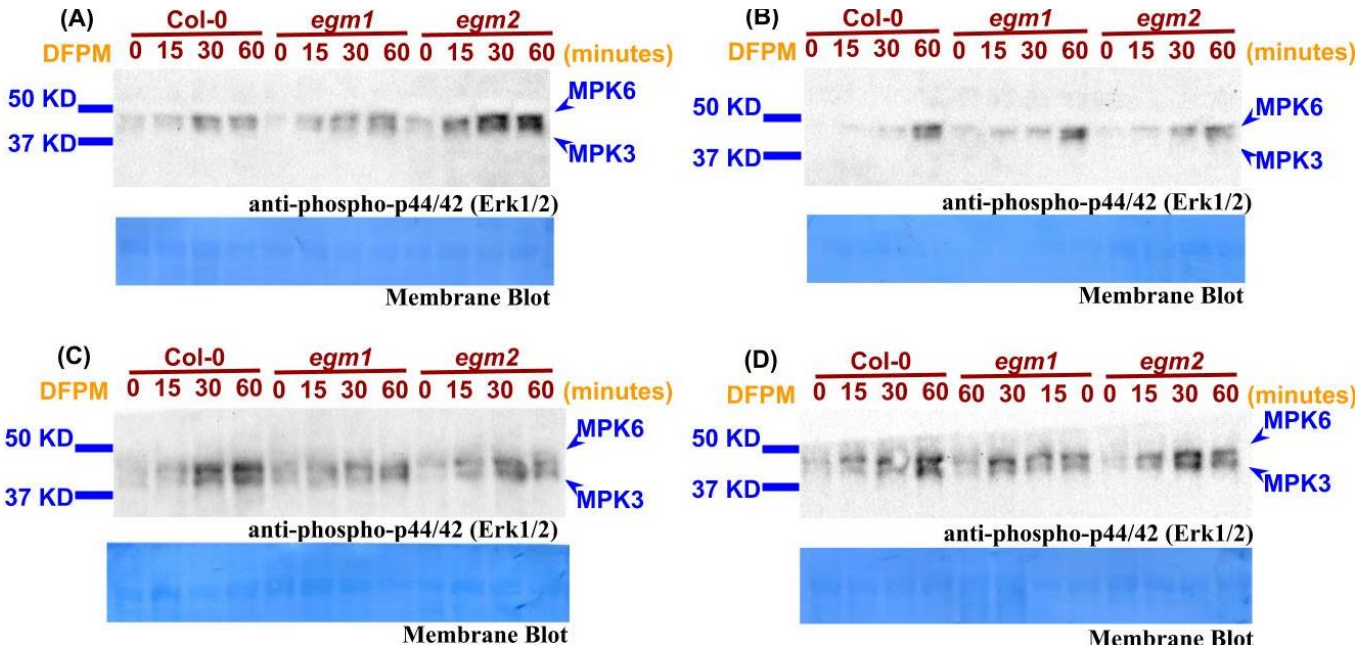


Figure 6a-d. DFPM-mediated activation of mitogen- activated protein kinases (MAPKs) between wild-type and the *egm1* & *egm2* single mutants.
Single mutants of the EGM genes do not show a clear difference in DFPM-mediated activation of MAPKs compared to Col-0. *EGM1* and *EGM2* do not appear to be essential for DFPM-mediated activation of MAPKs. Plants were treated with 30 µM DFPM for the indicated time points and used for whole protein extraction and subsequent Western blotting. Phosphorylated MAPKs were detected using anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) antibody. Loading controls: Coomassie blue staining of membranes. Results from four independent replicates (a-d) are shown.

DFPM-mediated MAPK-activation was compared between wildtype Col-0 and the egm-amiRNA line across four different time points. An increased response in MAPK-activation was observed in the *egm-amiRNA* line compared to Col-0 as shown by the stronger presence of MAPK proteins observed across three biological replicates after exposure to DFPM (Figure 7a-

c). When compared to the egm1 and egm2 mutant lines, the egm-amiRNA line shows a more enhanced phenotype. Since mutant lines of *RDA2* show a decreased response in DFPM-mediated MAPK-activation (Figure 5a-c), we speculate that the amiRNA silencing of *EGM1* and *EGM2* results in the upregulated expression of *RDA2* due to their proximity and homology to each other. Further testing of *EGM1*, *EGM2*, and *RDA2* is needed to test this hypothesis.

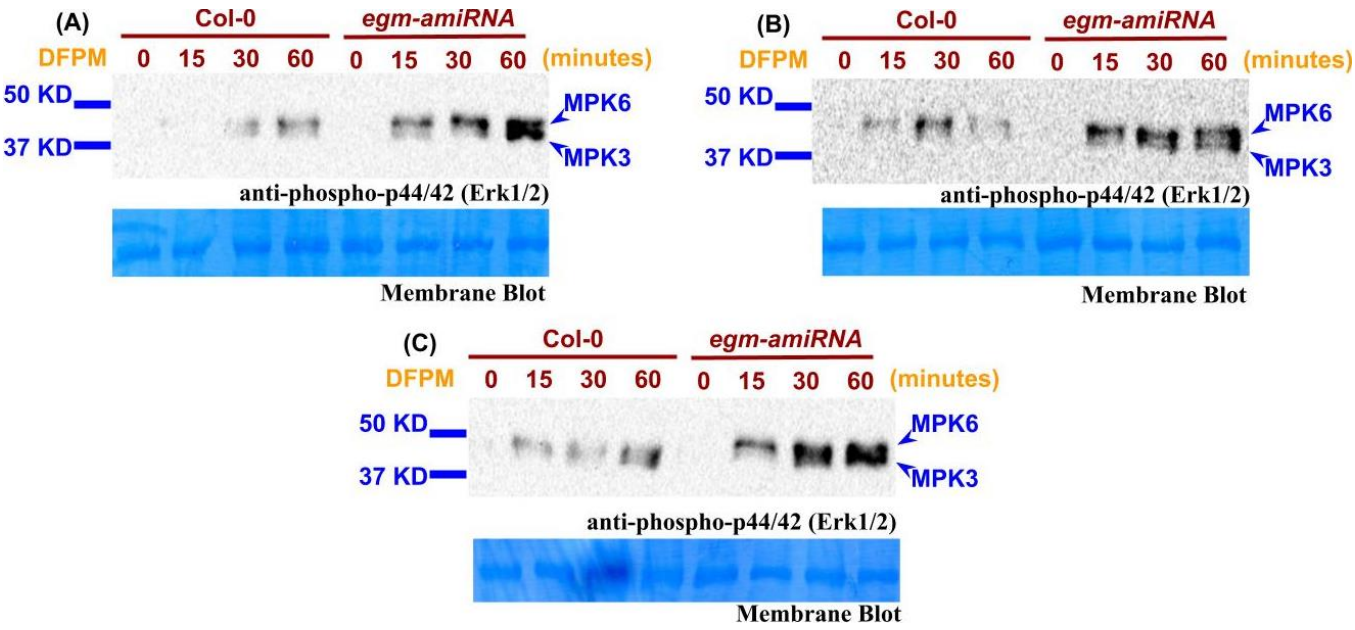


Figure 7a-c. DFPM-mediated activation of mitogen- activated protein kinases (MAPKs) between Col-0 and the egm-amiRNA line.
The egm-amiRNA line (which targets *EGM1* & *EGM2*) shows a stronger presence of DFPM-mediated activation of MAPKs compared to Col-0; it is possible that *RDA2* is being overexpressed in the *egm-amiRNA* line. Plants were treated with 30 µM DFPM for the indicated time points and used for whole protein extraction and subsequent Western blotting. Phosphorylated MAPKs were detected using anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) antibody. Loading controls: Coomassie blue staining of membranes. Results from three independent replicates (a-c) are shown.

3.5 *pRAB18::GFP* Fluorescent Expression Analysis of Near-Isogenic Lines

In addition to using qRT-PCR and DFPM-mediated MAPK-activation to characterize the function of VICTR-like genes, we attempted to quantify fluorescent expression of Col-0, NIL-Col-0 and NIL-Bu-5 using lines of these genotypes transformed with agrobacterium containing a

GFP promoter in the RAB18 locus (*pRAB18::GFP),* specified as an ABA reporter. The pRAB18-GFP reporter has been initially established to screen for chemical compounds that affect ABA signaling such as DFPM (Kim et al., 2011; Waadt et al., 2015; Park et al., 2019). Using confocal microscopy I analyzed relative fluorescence intensity of *pRAB18::GFP* in the abaxial leaf surface of my genotypes after chemical treatments to compare their response.

I attempted to compare the fluorescence intensity of Col-0 and the two NILs after exposure to DFPM, ABA, or their respective controls (0.04% DMSO, 0.02% ethanol). During my first non-blinded trial I observed an increase in fluorescence intensity for Col-0, NIL-Col-0, and NIL-Bu-5 under the ABA treatment compared to the control condition (Figure 8a-b) which is to be expected due to *RAB18's* role as an ABA reporter. Under the ABA & DFPM chemical treatment (pretreatment with DFPM and treatment with ABA) fluorescence intensity was comparable to wildtype for Col-0 and NIL-Col-0; NIL-Bu-5 on the other hand showed fluorescence intensity similar to its respective ABA treatment (Figure 8a-b). This difference in chemical response led us to speculate that the lack of *VICTR* and its homologous genes in NIL-Bu-5 contributed to its increased resistance to DFPM-inhibition of ABA when compared to its respective controls Col-0 and NIL-Col-0, suggesting that *VICTR, VICTL1,* and the two tandem TIR-NB-LRR's function together in the cross-talk between ABA and DPFM-signaling. During my second attempt to compare *pRAB18::GFP* fluorescent intensity between Col-0, NIL-Col-0, and NIL-Bu-5 I performed a double-blind assay (in which the genotypes and the chemical treatments were unknown to me during the time of microscopy and only revealed after the fluorescent analysis was completed. A *VICTR* knockout line containing the insertion for *pRAB18::GFP* was used to compare its response to NIL-Bu-5. The chemical responses between Col-0, NIL-Col-0, and *victr-pRAB18::GFP* all followed the same trend as in the first experiment where we see an increase in fluorescent intensity between ABA and control treatments and

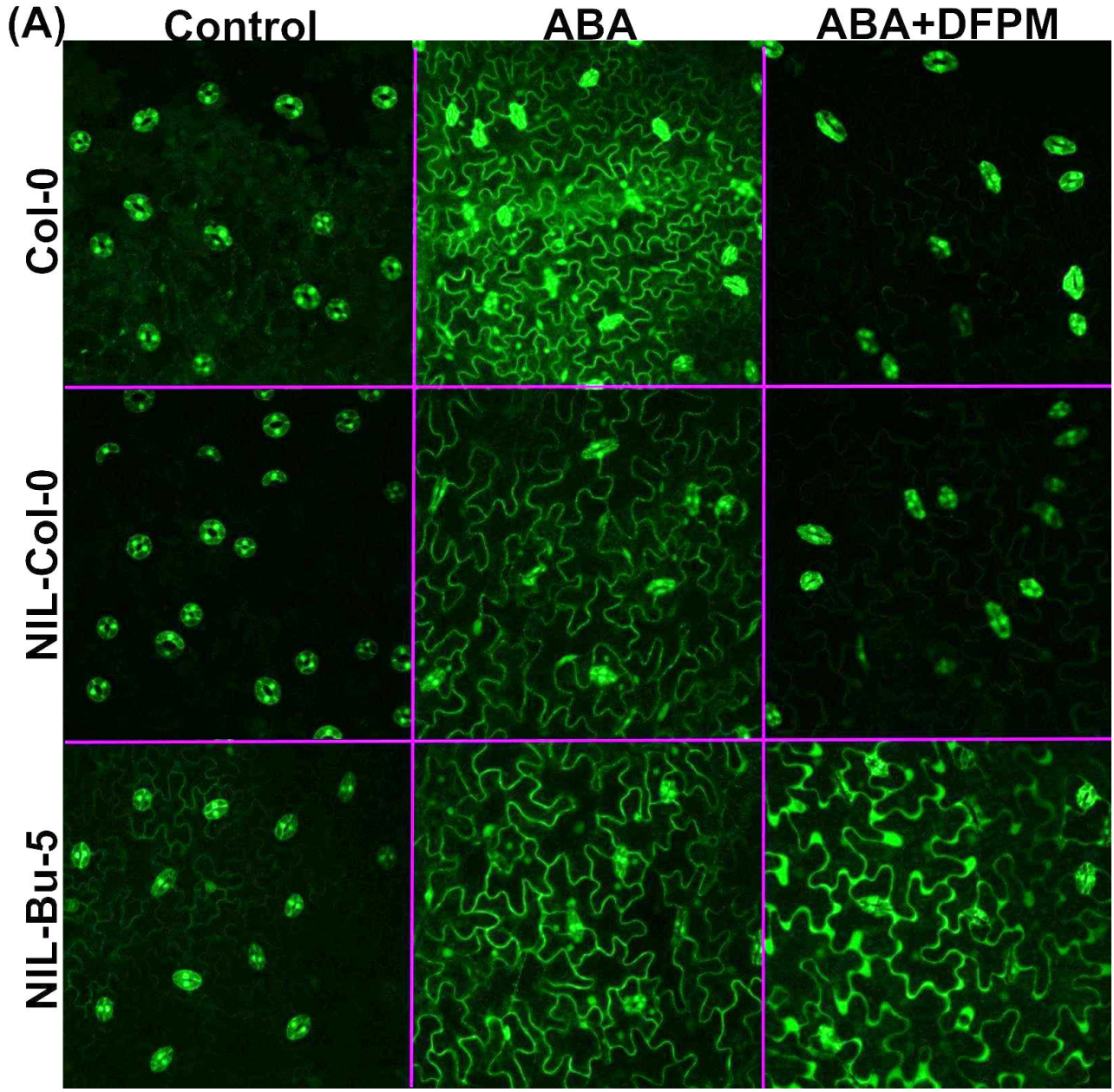


Figure 8a. Confocal imaging samples of Col-0, NIL-Col-0, and NIL-Bu-5 containing *pRAB18::GFP* after chemical treatments.
pRAB18::GFP fluorescence becomes more enhanced under the ABA chemical treatment as seen by the elevated signaling in the epidermal pavement cells when compared to control. Fluorescence intensity is inhibited in the presence of DFPM as shown by the decrease in fluorescent intensity when compared to ABA. Leaves of 14 day-old plants were pre-treated with 10 uM DFPM or solvent control (0.02% DMSO) for 1 hour followed by addition of 20 uM ABA or solvent control (0.04% ethanol). GFP fluorescence was observed from the abaxial leaf surface using confocal microscopy after 24 hours of ABA addition. Representative images from n = 3 biological repeats. Images displayed are 50% brighter than original captured images. Images were corrected for autoscale by scaling all the displayed images under the same range.

similar fluorescent intensity between control and ABA/DFPM chemical treatments (Figure 8b-c). With NIL-Bu-5 we saw that the fluorescence intensity is higher in the control compared to the ABA (Figure 8c). This seemed to suggest that the ABA uptake in NIL-Bu-5 was unsuccessful.

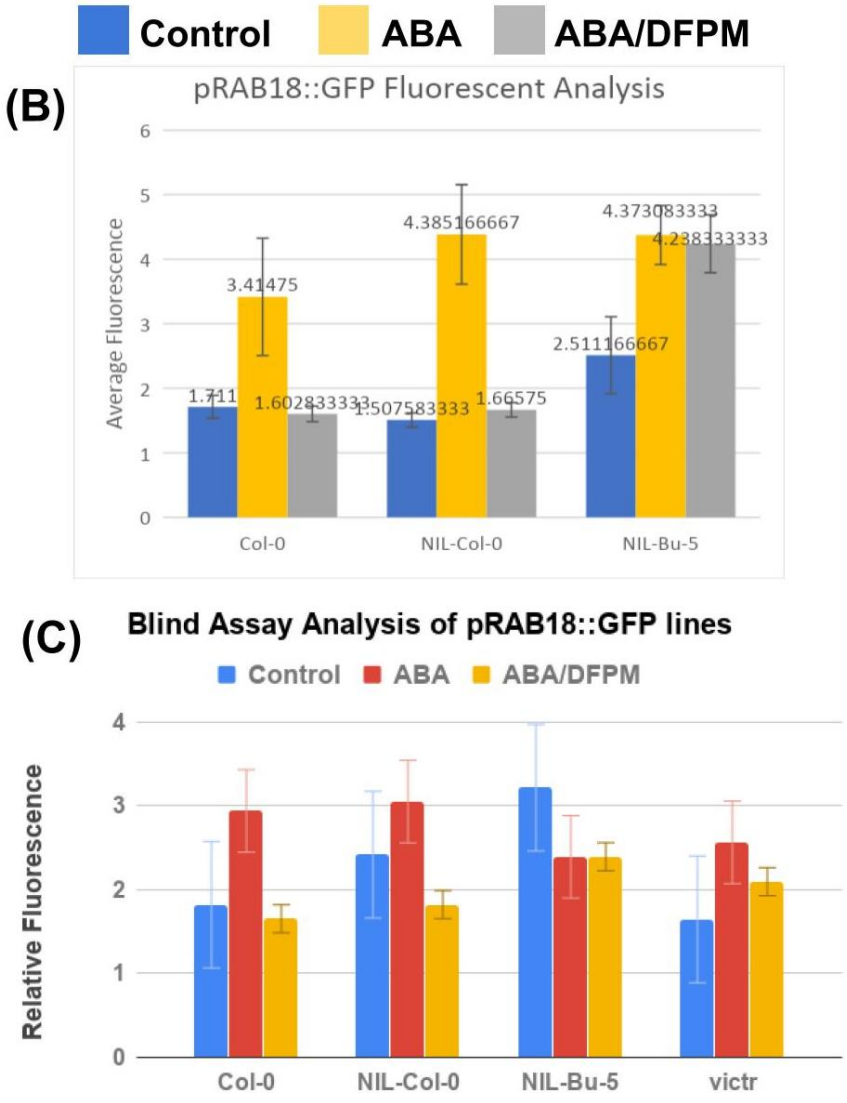
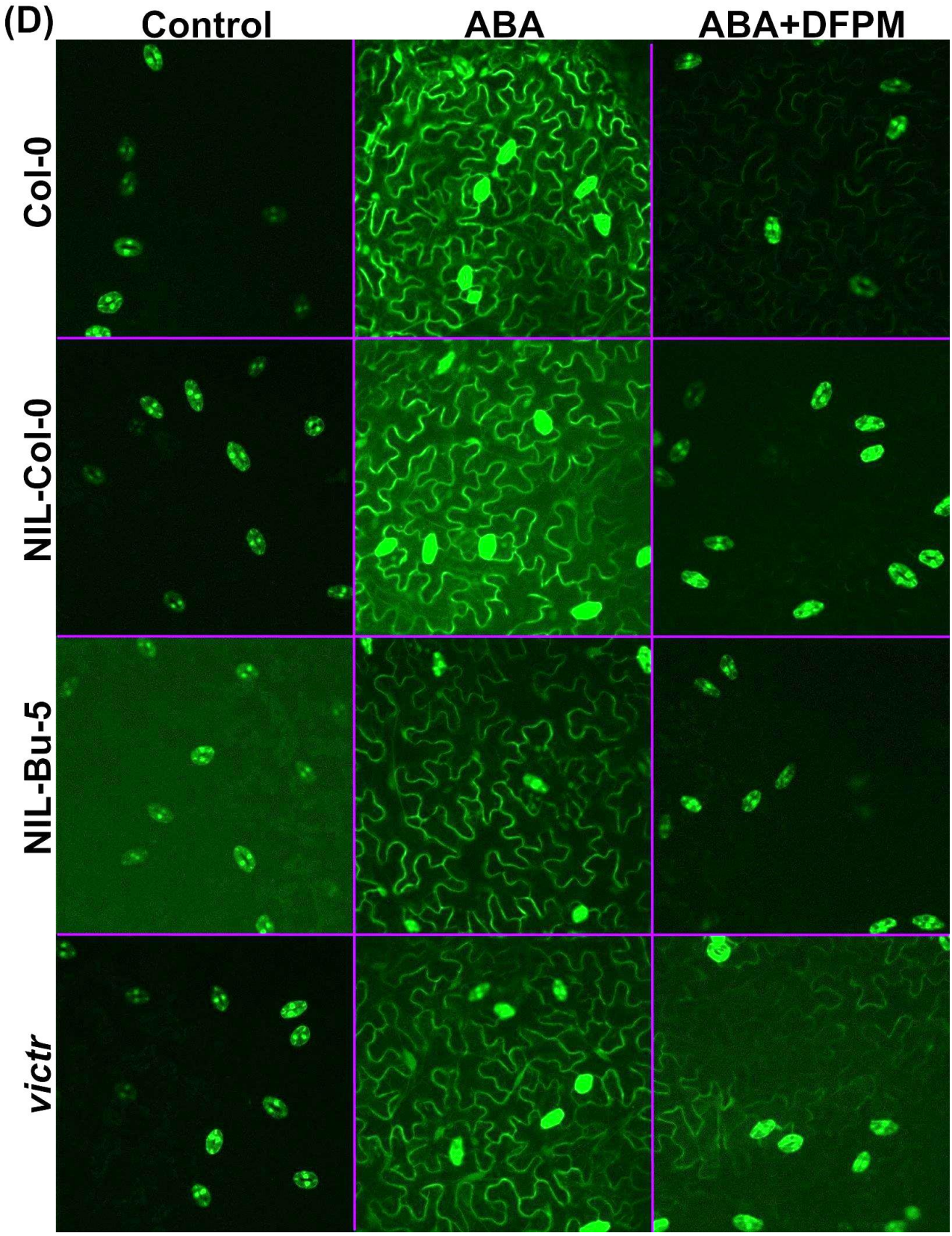


Figure 8b-c. Quantification of *pRAB18::GFP* fluorescence intensity for non-blinded (b) and blinded (c) trials.
Intensity of fluorescence signaling in Col-0, NIL-Col-0, NIL-Bu-5, and *victr* containing GFP (blind-assay only) under control, ABA and ABA/DFPM chemical treatments were quantified using ImageJ. In the non-blinded trial ABA-mediated gene expression in NIL-Bu-5 was less inhibited by DFPM compared to Col-0 and NIL-Col-0. In the blind assay trial NIL-Bu-5 control shows higher fluorescent intensity. Further blind assay experiments are needed. Results are an average of n=3 biological replicates. Error bars = SEM.

Figure 8d (following page). Confocal imaging samples of Col-0, NIL-Col-0, NIL-Bu-5, and victr containing *pRAB18::GFP* after chemical treatments (double-blind trial).
pRAB18::GFP fluorescence becomes more enhanced under the ABA chemical treatment when compared to control for Col-0, NIL-Col-0, and *victr*. Fluorescence intensity is highest for NIL-Bu-5 under the control condition is shown by the increased intensitiy in the image background. Fluorescent intensity generally becomes weakened in the presence of DFPM as shown by the decrease in fluorescent intensity when compared to ABA for Col-0, NIL-Col-0, and victr. Fluorescence intensity is comparable between ABA and ABA/DFPm treatments in NIL-Bu-5 despite the presence of epidermal pavement cells. Leaves of 14 day-old plants were pre-treated with 10 uM DFPM or solvent control (0.02% DMSO) for 1 hour followed by addition of 20 uM ABA or solvent control (0.04% ethanol). GFP fluorescence was observed from the abaxial leaf surface using confocal microscopy after 24 hours of ABA addition. Representative images from n = 3 biological repeats. Images displayed are 30% brighter than original captured images. Images were corrected for autoscale by scaling all the displayed images under the same range.


(D)
Control
ABA
ABA+DFPM
Col-0
NIL-Col-0
NIL-Bu-5
victr

IV. Discussion

4.1 The role of VICTR-like genes in signaling transduction

The small molecule DFPM seems to function in two distinct signaling-pathways: (1) DFPM-mediated inhibition of abscisic acid (ABA) signaling via activation of Pathogen-triggered immunity (PTI)-signaling, and (2) DFPM-mediated root growth arrest via activation of VICTR (*Variation in Compound Triggered Root growth response)* (Kim et al., 2011; 2012, Kunz et al., 2016; Figure 1). My objective was to investigate the role of *VICTR* and its tandem homologs (*VICTR-like* genes) in the regulation of ABA after exposure to DFPM. To test for this I made use of the previously generated near-isogenic lines (NILs) NIL-Col-0 and NIL-Bu-5 (Ariga et al., 2017) along with wild type Col-0 to compare responses across all three genotypes after exposure to chemicals such as ABA, DFPM, and ABA/DFPM. *VICTR* and its three homologous genes are present in NIL-Col-0 but absent in NIL-Bu-5 (Ariga et al., 2017; Figure 2). These NILs will allow us to explore the role of VICTR with respect to the ABA and DFPM-signaling pathways.

In my first experiment I performed a qRT-PCR analysis in order to compare expression levels of ABA-reporter genes RAB18, RD29A, and ERD10 in the genotypes of Col-0, NIL-Col-0, and NIL-Bu-5 after exposure to ABA, DFPM, and ABA/DFPM chemical treatments (Figure 3a-c). All three genotypes follow a similar trend in ABA-induced gene expression: an increase in expression under ABA, expression comparable to control under DFPM, and an increased but impeded expression under the ABA/DFPM treament. These data seemed to suggest that VICTR and it's three homologous genes do not play a role in the down-regulation of ABA-signaling via suppression by DFPM.

The pathogen-defense signaling components EDS1, PAD4, SGT1b, and RAR1 are required for DFPM-mediated inhibition of ABA (Kim et al., 2011; 2012). DFPM can also be utilized to induce activation of mitogen-activated protein kinases (MAPKs) MPK3 and MPK6 in

a manner similar to flg22-mediated MAPK activation (Park et al., 2019; Asai et al., 2002). Activation of MPK3 and MPK6 is an example of an early signal response for recognition of pathogen or pathogen-induced molecules in PTI signaling (Meng and Zhang, 2013). Using NIL-Col-0 and NIL-Bu-5, I sought to investigate whether the presence or absence of VICTR and its three homologous genes contribute to the activation of MAPKs after exposure to DFPM.

I observed no clear differences in the levels of phosphorylated MPK3 and MPK6 across four different time points after exposure to DFPM between Col-0, NIL-Col-0, and NIL-Bu-5. This result was observed across four different biological replicates (Figure 4a-d). Because we saw no discernible difference between NIL-Bu-5 and its respective control NIL-Col-0, these data seemed to suggest that VICTR and its close tandem-repeat homologs are not involved in DFPM-mediated activation of MAPK proteins MPK3 and MPK6 (Park et al., 2019).

4.2 The role of RDA2 in activation of MAPKs/Immune responses

The Lectin Receptor Kinase (LecRK) *RDA2* (***R****ESISTANCE TO* ***D****FPM-INHIBITION OF* ***A****BA SIGNALING****2)*** was identified through a chemical genetics screening and found to be required for inhibiting ABA-signal transduction after exposure to DFPM (Park et al., 2019). Two mutant alleles of *RDA2*, *rda2-1* and *rda2-2*, were generated through EMS-mutagenesis and T-DNA insertion respectively (Park et al., 2019). I sought to compare DFPM-mediated activation of MAPKs between the two rda2 mutants and their respective wildtype control.

rda2-1 was generated in the background of Col-0 with *pRAB18::GFP* whereas *rda2-2* was made in a non-transformed Col-0 background (Park et al., 2019). *rda2-1* and *rda2-2* both showed a diminished response in the activation of MPK3 and MPK6 after exposure to DFPM when compared to their respective wildtype (Figure 5a-c). This seems to suggest that the RDA2 LecRK is involved in early DFPM-signaling and transduces downstream signaling pathways such as MAPK activation (Park et al., 2019). Seeing as activation of MPK3 and MPK6 are

dependent on RDA2 it is possible that both MAPKs are directly responsible for repressing ABA-induced signaling and are activated by DFPM via RDA2 (Figure 1). Further testing of higher order mutants would be needed to verify this hypothesis.

4.3 Possible regulation of RDA2 by EGM1 and EGM2 tandem homologs

Two genes that lie upstream of *RDA2*, *EGM1* and *EGM2 (ENHANCED SHOOT GROWTH UNDER MANNITOL STRESS)* encode for LecRKs that are thought to be involved in biotic stress response mediated by exposure to mannitol, secreted by fungal pathogens (Trontin et al., 2014; Velez et al., 2008). Two T-DNA mutants targeting *EGM1* and *EGM2*, *egm1* and *egm2*, were generated along with an artificial-microRNA line that targets both *EGM* genes (*egm-amiRNA*; Trontin et al., 2014). Using T-DNA single mutants: *egm1, egm2,* and the *egm-amiRNA* line I sought to investigate the role of the *EGM* genes in the activation of MAPKs after DFPM exposure to explore any potential connection to *RDA2*.

When comparing the *egm1* and *egm2* T-DNA mutants to wildtype Col-0 I observed activation of MAPKs MPK3 and MPK6 across four different time periods between the three genotypes shown in four different biological replicates (Figure 6a-d). These findings contrast the measurable impairment observed in the previous experiment with the allelic mutants of *rda2*. From these data it seems that *EGM1* and *EGM2* individually do not play a role in DFPM-mediated activation of MAPKs. I performed the same experiment comparing the *egm-amiRNA* line targeting both EGM genes and found that there was a stronger presence of MPK3 and MPK6 across four different time points after activation of MAPKs by DFPM when compared to wildtype Col-0 (Figure 7a-c). This result was observed in three biological replicates which led us to speculate that perhaps *EGM1* and *EGM2* are both involved in co-regulating *RDA2* and *RDA2* becomes overexpressed when both genes are targeted due to their proximity & homology. Further testing of higher order mutants would be needed to test this hypothesis.

4.4 Additional microscopy and blind assay trials

One result that I was unable to successfully replicate was a microscopy assay to analyze the levels of *pRAB18::GFP* fluorescent expression in Col-0, NIL-Col-0, and NIL-Bu-5 after exposure to various chemical conditions. Each line was transformed with agrobacterium containing *pRAB18::GFP* (Zhang et al., 2006) and seeds were screened for GFP fluorescence. Once each line was established I performed a chemical-microscopy assay by exposing each genotype to control, ABA, or ABA/DFPM chemical treatments to compare the fluorescent response of each genotype. In my initial screening I found that Col-0 and NIL-Col-0 were not resistant to DFPM-mediated inhibition of ABA, as shown by the similar fluorescent expression between control and ABA/DFPM treatments, but that NIL-Bu-5 was resistant as shown by the similarity in fluorescent expression between the ABA and the ABA/DFPM chemical treatments (Figure 8a-b). This result led us to believe that perhaps *VICTR* and its tandem homologs play a role in the crosstalk interaction between ABA and DFPM signaling and that the absence of the four *VICTR* genes in NIL-Bu-5 contributed to its increased resistance to DFPM.

I attempted to replicate this same result using a double-blinded assay in which the genotypes were relabeled and the chemical conditions were performed by someone else. This way we would remove any unintentional confirmation bias from the experiment and I would perform the microscopy imaging and analysis without knowing the genotype or chemical treatment of each sample. During this blind-assayed trial I also included a mutant line of VICTR containing the *pRAB18::GFP* insertion, *victr-pRAB18::GFP*, as an additional control for NIL-Bu-5. In the blind-assay trial the results for Col-0 and NIL-Col-0 were similar in that they both show increased fluorescence under ABA and fluorescence similar to the control treatment under the ABA/DFPM condition (Figure 8c). *victr-pRAB18::GFP* also showed this same trend as Col-0 and NIL-Col-0 (Figure 8c). NIL-Bu-5 on the other hand showed decreased expression of

pRAB18::GFP fluorescence under the ABA and ABA/DFPM chemical treatments when compared to the control condition (Figure 8c), contradictory to our previous results. This seems to suggest that the ABA uptake was unsuccessful in NIL-Bu-5. It could be noted that the absence of the four VICTR genes of NIL-Bu-5 could result in a diminished capability to uptake ABA.

In order to address these differing results additional double-blinded microscopy trials are needed. One suggestion is to perform the experiment in the span of multiple days in order to gather data in small replicates since the lack of focus or exhaustion as the day continues could contribute to differing images quality used in the analysis. Having another person perform the analysis besides the experimenter could also help in validating our double-blind approach.

4.5 Future Directions & Questions of Interest

In conclusion, the present data in this thesis seems to suggest that *VICTR* and its close homologs while playing a very important role in DFPM-induced root growth arrest (Kim et al., 2012; Kunz et al., 2016) are not necessarily involved in DFPM-inhibition of ABA signaling. This is shown by the similarities in ABA-induced gene expression between Col-0, NIL-Col-0 and NIL-Bu-5 (Figure 3a-c). We also observed that the four VICTR genes are not involved in DFPM-mediated activation of MAPKs (Figure 4a-d). Although inconclusive it is possible that the lack of the four VICTR genes in NIL-Bu5 could affect the expression of the *pRAB18::GFP* promoter (Figure 8a-c). Using both NIL-Col-0 and NIL-Bu-5 as a tool future characterization of each individual TIR-NB-LRR gene found within the VICTR-locus could take place.

RDA2 was identified through a chemical genetic screen and shown to be involved in DFPM-mediated inhibition of ABA as well as DFPM-mediated activation of MAPKs (Park et al., 2019; Figure 5a-c), Two genes in tandem to RDA2, EGM1 & EGM2, were shown to be invovled in mannitol-induced stress (Trontin et al., 2014) and may be involved in co-regulating expression of RDA2 via increased activation of MPK3 and MPK6 (Figure 7a-c). It would be

interesting to further explore the relationship between RDA2, EGM1, and EGM2 by performing experiments with higher order mutants of these three genes as well as investigate whether EGM1 and EGM2 play a role in DFPM-mediated inhibition of ABA signaling, similar to RDA2.

Chemical genetics is a powerful tool for helping to understand signaling pathways. Through this analysis it is possible that our new understanding of stress-signaling pathways may help contribute to the success of generating environmentally-adaptive crops in the future.

V. References

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