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Insights into Abscisic Acid and Phosphatidic Acid Signal Transduction Dynamics in Arabidopsis thaliana

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INSIGHTS INTO ABSCISIC ACID AND PHOSPHATIDIC ACID SIGNAL TRANSDUCTION DYNAMICS IN ARABIDOPSIS THALIANA

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

The Department of Biological Sciences

by
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B. ED (Sci)., Kenyatta University, 2008
December 2021
This work is dedicated to my son Linus Kihara and to my parents Regina Wairimu and the late Daniel Ndathe.
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ABSTRACT

Abscisic acid (ABA) is a plant hormone that triggers a signaling transduction in response to drought, salinity, and cold. The core components in the ABA signaling pathway that lead to downstream ABA responses have been identified.

Phosphatidic acid (PA) is a membrane phospholipid that is involved in the signaling pathway for ABA-induced stomatal closure. We tested whether PA is involved in the signaling pathway for ABA-induced gene expression as well, using model plant Arabidopsis thaliana. We selected the RD29A gene which is a canonical drought gene that is induced by ABA. We found that PA is not involved in the signaling pathway for ABA-induced gene expression even though core components involved in the ABA signaling pathway are shared between stomatal closure and gene expression. We propose that some of the homologous phosphatases that are not inhibited by PA, lead to ABA-induced gene expression.

To advance the finding that PA is involved in the signaling pathway for ABA-induced stomatal closure, we created a PA biosensor based on dimerization-dependent fluorescent proteins. The biosensor allowed us to determine the spatiotemporal production of PA in Arabidopsis plants when exposed to ABA or sodium chloride (NaCl). The PA biosensor showed that there was an increase in PA concentrations with ABA and NaCl on the plasma membrane within 10 minutes. On the internal membranes, PA concentrations increased on exposure to ABA but not to NaCl. These results suggest that the formation of transient PA on different membranes is specific to the stress.

To further investigate the connectivity of core components in the ABA signaling pathway and identify the next research frontiers in the ABA-signaling pathway, we constructed a dynamic model with a set of ordinary differential equations. Computational analysis predicted that the
translation rate constant of protein phosphatases (PP2Cs), which are negative regulators in the pathway, determine the kinetics of ABA-mediated gene expression. Changes in the PP2C translation rate constant altered the levels of gene expression and the peak time point of expression. We propose that studying alteration of translation rates by ABA will help grow plants that are tolerant to drought.
CHAPTER 1.
INTRODUCTION

1.1 Drought stress in plants

Drought stress in plants refers to a condition where plants are unable to obtain water due to low water potential in growth media in comparison to the root cells which can be described as a soil-water deficit. This could be because of lack of water availability in the soil due to reduced precipitation or the inability of the plants to absorb water from the soil brought about by low water potential also known as physiological drought. Low water potential in the soil is usually due to high salinity or extremely low temperatures leading to freezing of water. Drought could also be caused by increased evapotranspiration due to elevated temperatures that not only lead to increased evaporation of water from the soil but also loss of water by the plant through transpiration, hence the name (Hossain et al., 2016), (Mahmood et al., 2019), (Bray, 1997).

1.2 Current global status of drought and the projected future

The Intergovernmental Panel on climate change (IPCC) 2013 reported that global temperatures were on the rise with a reported increase of 0.85°C between 1880 and 2012. (AR5 Climate Change 2013: The Physical Science Basis — IPCC). Due to this, regions in drier areas were reported to be drier while regions in wet were wetter (Feng and Zhang, 2015). For example, the Mediterranean, west and central Asia, and southern Africa saw an increase in air temperatures that in turn increased the frequency and intensity of drought incidences as reported in (IPCC special report: Impacts of 1.5°C Global Warming on Natural and Human System). The increased air temperatures led to increased evapotranspiration and reduced precipitation causing drought. Global temperatures continue to increase and therefore drought incidences will also increase in the future.
Drought has adverse effects on agriculture including reduced crop and livestock productivity and increased cost of doing agriculture due to the need for irrigation (Cline, 2003). This in turn reduces food availability and hence reduced food security. The regions mentioned above include developing countries that have a lower capacity to adapt to the changes in climate (Ilyas et al., 2020), (Cline, 2003).

Global drought effects will be exacerbated by increased global population especially in developing countries further reducing food security. The world population is expected to increase from 7.7 billion currently to 9.7 billion by the year 2050 and much of the population growth will be in Africa (Brears, 2021). This means developing African countries like Kenya, my home country, face a double challenge. The first challenge is a projected increase in global temperatures therefore adverse effects on agriculture, resulting in low food availability. The second challenge is increased population and a subsequent rise in demand for food. In most developing countries, agriculture is also a means of income, and therefore poor crop productivity also means a loss of livelihood (Luo, 2010), (Drought and Agriculture | Land & Water | Food and Agriculture Organization of the United Nations | Land & Water | Food and Agriculture Organization of the United Nations).

1.3 Situation in Kenya as an example of a country that experiences frequent drought

Kenya, a developing nation in Africa, has a population of over 54 million (Kenya Population (2021) - Worldometer.) and agriculture is the backbone of the economy. Agriculture contributes to about 26% of the Kenyan GDP directly. Drought incidences are prevalent in Kenya especially in the arid and semiarid regions which constitute 80% of the landmass (Kenya: Drought - 2014-2021. ReliefWeb). The drought incidences occur in cycles every few years. Previously, the drought would occur every 10 years but in the recent past, the frequency has increased to every 3
to 5 years affecting millions of Kenyans and their livestock (Opiyo et al., 2015), (Huho and Mugalavai, 2010). The World Bank reported that there was an average of 0.21°C increase in temperature per decade since the 1960s and there was reduced overall annual rainfall affecting the two rain seasons. (World Bank Climate Change Knowledge Portal), (Why Kenya’s seasonal rains keep failing and what needs to be done - Kenya. ReliefWeb). Increased frequency of drought is a major problem in Kenya since most of the agriculture depends on rain. For example, during the 2017 drought, 2.7 million Kenyans were at risk of hunger and most needed relief food for sustenance (Kenya: Drought - 2014-2021. ReliefWeb). These unpredictable weather patterns are projected to continue, and this leaves most of the Kenyan population at risk from drought. On the other hand, the Kenyan population is on the rise and projected to reach over 90 million by the year 2050 (Kenya Population (2021) - Worldometer). With these, urgent measures are required to mitigate the effects of drought on agriculture, to reduce their impact on the population and enhance food security.

1.4 Effects of drought on plants

One way to mitigate drought effects on agriculture is to understand how plants respond to drought stress and therefore find ways of creating more drought-resistant plants. Plants employ three methods to withstand drought, either escape, avoidance, or tolerance. In escape, plants reproduce faster or flower early and reproduce before the onset of drought and therefore the plant does not experience drought, although this leads to reduced yields. In avoidance, the plant uses different mechanisms to maintain a high-water content or maintain a low osmotic potential in the cells e.g., reducing the stomata size and increasing the root network system. This helps the plant absorb water and transport it up the plant. In tolerance, the plant maintains normal activity in the low water content. It involves mechanisms for osmotic adaptation, an increase in antioxidant
activity, and altering different metabolic pathways (Mahmood et al., 2019), (Bray, 1997), (Luo, 2010), (Gupta et al., 2020).

The methods of response create different effects on plants including physiological, cellular, molecular, and morphological. Drought leads to reduced cell expansion due to loss of turgor in cells. Plants also exhibit higher root growth to increase water absorption but, on the other hand, there is reduced shoot growth and overall reduced plant growth. In addition, stomatal closure and reduced number of stomata help reduce transpiration (Mahmood et al., 2019). All these have the overall effect of reducing the rate of photosynthesis in addition to reducing the concentration of chlorophyll. However, xanthophylls have been shown to increase in plants experiencing drought stress since they protect the plant from oxidative damage (Hossain et al., 2016), (Kumar et al., 2018). Drought also leads to disruption of the membranes which leads to reduced ion uptake (Lauriano et al., 2000). These responses take place due to elaborate signaling pathways with numerous signaling molecules.

1.5 Drought response signaling in plants

Plants being sessile organisms have developed extensive mechanisms involved in the detection and response to drought. Like many other stimuli, drought is detected by signaling receptors which in turn relay the signal to second messengers that then induce a signal response to alleviate the drought. Second messengers in the signaling pathway include calcium ions (Yang and Poovaiah, 2003), hormone abscisic acid (ABA) (Finkelstein, 2013), reactive oxygen species (ROS) (Dat et al., 2000), phospholipids such as inositol phosphates (Munnik and Vermeer, 2010), and phosphatidic acid (Testerink and Munnik, 2005). Once the signaling pathway has been induced, distinct types of responses or output are created.
Protein phosphorylation is an important aspect of signal transduction and different kinases have been shown to be activated and mediate the response during drought or osmotic stress. These include mitogen-activated protein kinases (MAP kinases) (Sinha et al., 2011), sucrose non-fermenting 1 (SNF1) related kinases (Fujita et al., 2009), and calcium-dependent protein kinases (CDPK/CPK) (Campo et al., 2014). The kinases activate other proteins leading to downstream responses like gene expression, stomatal closure, etc. (Dubrovina et al., 2015), (Fujita et al., 2009).

In response to the signaling pathway, different effectors are produced to mitigate the drought stress. Most of the effectors are proteins encoded by drought-responsive genes. Stress response genes are classified as either functional or regulatory (Agarwal et al., 2017). Functional genes encode enzymes required in the synthesis of osmolytes, ion transport proteins, enzymes important for the synthesis and degradation of ROS, and lipid biosynthesis proteins. Regulatory genes encode protein receptors, protein kinases, and transcription factors (Xiong et al., 2002), (Zhu, 2002).

### 1.5.1 Types of signaling molecules involved in drought signaling

**Ca^{2+} ions.**

Calcium is an essential macronutrient required for plant growth and other metabolic processes like mitosis. In plant cells, it is kept at concentrations of sub-micro molar range and an increase in the cytosolic concentrations elicits a signal (Tuteja, 2009). It is also a versatile second messenger whose cytosolic concentration increases due to drought (Knight et al., 1997). This phenomenon is created due to either Ca^{2+} influx through transporters into the cell via the plasma membrane or via the action of Ca^{2+} efflux pumps that pump Ca^{2+} from organelles like the endoplasmic reticulum or the vacuole where Ca^{2+} is stored. The increase in cytosolic Ca^{2+} concentration acts as a signal that is transduced via calmodulin, calcium-dependent kinases, or other calcium-regulated proteins. All of this leads to other downstream responses e.g., expression
of stress-induced genes. An increase in $\text{Ca}^{2+}$ is transient and soon after signal induction, the ions are sequestered back into the organelles to terminate the calcium signal and restore normal concentrations of the ion (Knight, 1999).

**Reactive oxygen species (ROS).**

Drought induces the production of ROS species e.g., superoxide ($\text{O}_2^-$), hydrogen peroxide ($\text{H}_2\text{O}_2$), singlet oxygen ($^1\text{O}_2$), and hydroxyl radicals (OH’) (Xiong et al., 2002), (Cruz de Carvalho, 2008). The concentration of ROS changes due to a delicate balance between ROS-producing enzymes e.g., NADPH oxidases like the respiratory burst oxidase homolog (RbohD) proteins versus ROS scavengers. ROS at high concentrations is deleterious to the cell and causes oxidative damage that inhibits cellular reactions like photosynthesis. Therefore, plants have elaborate mechanisms to remove excess ROS from the cell through ROS scavenging enzymes like catalase and peroxidase or antioxidant compounds like ascorbic acid and carotenoids (Hussain et al., 2019), (Cruz de Carvalho, 2008). ROS is also involved in drought-related signal transduction and hence its increase in exposure to drought (Dat et al., 2000). ROS is important in the activation of calcium channels in guard cells, participates in calcium signaling cascades, stomatal closure, and has been shown to induce expression of drought-induced gene dehydration-responsive element-binding protein 2a (DREB2A) which encodes a transcription factor important in ABA-independent gene expression (Zhu, 2002).

**Protein kinases.**

Protein kinases are central in the sensing and relaying signal responses in plants. Protein kinases catalyze the transfer of a phosphate to a protein thus altering its activity and this then creates downstream responses. During drought or osmotic stress, different protein kinases have been reported to play a role in the signaling responses. They include (SNF1)-related protein kinases.
(SnRKs), MAP kinases, receptor-like kinases (RLKs), and CDPKs/CPKs (Chen et al., 2021). SnRK kinases consist of three subfamilies in plants SnRK1, SnRK2, and SnRK3 with 38 members. The SnRK2 sub-family consisting of 10 members is activated by osmotic stress and some by ABA (Boudsocq et al., 2007). Recently, ABA-activated SnRK2 kinases have been shown to be central in the ABA signaling pathway and triple gene knockout mutants of snrk2.2/3/6 were found to be ABA insensitive (Coello et al., 2011). MAP kinases consist of three types of kinases that form a cascade. They are MAP kinase kinase kinases (MAPKKK) that activate MAP kinase kinases (MAPKK) which in turn activate MAP kinases forming a chain reaction (Chen et al., 2021). Studies have shown that these kinases are involved in ABA signaling and response to drought in plants e.g., MAPKKK18 overexpression in Arabidopsis plants enhanced drought stress resistance while the respective knockout mutant was drought-sensitive (Li et al., 2017).

**Protein phosphatases.**

Protein phosphatases are important molecules in signaling pathways that utilize kinases since they help to maintain a normal balance in the cell by dephosphorylating proteins and hence turn off the signal. There are diverse types of phosphatases in plants dependent on the type of amino acid they dephosphorylate i.e., serine/threonine and tyrosine phosphatases (Luan, 1998). Serine/threonine phosphatases can further be classified into the following groups: protein phosphatase 1/2A/2B/4/5/6/7/2C (PP1, PP2A, PP2B, PP4, PP5, PP6, PP7, and PP2C) whose activity is dependent on the presence of Mg\(^{2+}\). In plants, PP2C phosphatases are more prevalent and most of their genes were shown to be differentially expressed during drought (Singh and Pandey, 2012). Moreover, some members of the PP2C family were determined to be negative regulators of the ABA signaling pathway (Rubio et al., 2009).
Lipids.

Lipids are major structural components of biological membranes including the plasma membrane which is the boundary of the cell and plays a role in the perception and transmission of stimuli. Several lipids have been characterized as signaling molecules and they are found in different classes such as phosphoinositides, sphingolipids, phosphatidic acid, diacylglycerol, free fatty acids, oxylipins, and lysophospholipids (Wang and Chapman, 2013). Some of these have been shown to be important in drought response signaling. For example, dehydration induces the production of phosphatidic acid, phosphoinositides, oxylipins, and sphingolipids. Synthesis of these lipids is transient and initiates downstream responses to the signal (Hou et al., 2016).

Phytohormones.

Drought has also been shown to alter the synthesis of various phytohormones including abscisic acid (ABA), salicylic acid (SA), jasmonic acid (JA), ethylene (ET), Auxin (AUX), gibberellins (GA), brassinosteroids (BR), and cytokinins (CK). ABA is the predominant stress hormone and is involved in stress signal transduction. ET and AUX responsive genes have been shown to increase on the onset of drought stress while JA enhances drought resistance by playing a role in stomatal closure and ROS scavenging (Munemasa et al., 2011), (Jung et al., 2015), (Liu and Zhang, 2017). Studies on both SA and CK have reported both negative and positive effects on drought resistance (Prerostova et al., 2018), (Xiong et al., 2002), (Miura and Tada, 2014). BR enhance drought stress responses through ABA signaling molecules including ABA insensitive 1/2 (ABI1) and ABI2 (Ullah et al., 2018). In addition, BR signaling negative regulator brassinosteroid-insensitive 2 (BIN2) dephosphorylates SnRK2 kinases which are important in the ABA signaling pathway (Gupta et al., 2020). However, GA is reported to be a negative regulator in drought responses. Plants with low GA synthesis show higher drought tolerance while ectopic
expression of GA inactivator GA 2-oxidase (GA2ox) was shown to enhance drought tolerance (Ullah et al., 2018).

1.5.2 Drought response effectors

Drought-induced genes.

Several genes are induced as responses to drought stress. These genes can broadly be characterized as either early response or delayed response genes. Early response genes expression is induced within minutes of detection of the stimuli and usually encodes transcription factors that regulate the expression of the delayed response genes. Examples of early response genes include dehydration responsive element binding proteins (DREBs) and ABA-responsive element binding factors (AREBs) or ABRE binding factors (ABFs) (Hoang et al., 2017). Delayed response genes are expressed hours after the onset of the stimulus and are important in initiating drought tolerance in the plant. They include Responsive to drought 29A/29B/22/19 (RD29A/29B/22/19), cold-regulated 15/47 (COR15/47), kinases1/2 (KIN1/2), responsive to ABA 18 (RAB18), etc. (Xiong et al., 2002), (Zhu, 2002). All drought inducible genes have dehydration responsive element (DRE) or ABA-responsive element (ABRE) on their promoters that allow their expression to be regulated in response to drought or ABA (Zhu, 2002).

Osmolytes.

Osmolytes including proline, glycine-betaine, polyamines, and sugars like trehalose, mannitol, and sorbitol increase inside the cell on the onset of drought stress (Darko et al., 2019). These molecules are important during osmotic stress since they help in maintaining high osmotic pressure in the cytosol allowing the cells to maintain turgor pressure (Sharma et al., 2019). The osmolytes also protect the plant cells from oxidative damage by ROS which is produced during drought (Sharma et al., 2019).
**LEA proteins.**

Late embryogenesis abundant (LEA) proteins were first described to be expressed during seed maturation in the formation of the embryo hence the name (Olvera-Carrillo et al., 2011). However, it was later discovered that the same type of proteins were synthesized in vegetative tissues in response to water deficit and are important in water stress tolerance (Tunnacliffe and Wise, 2007). They are hydrophilic and are hypothesized to act as membrane and protein stabilizers during osmotic stress (Battaglia et al., 2008).

**1.6 ABA the stress phytohormone**

ABA is one of the classical phytohormones first described in the 1960s as abscisin II (hence its name) since it was found to promote leaf abscission in cotton (Ohkuma et al., 1963). It was also described by other groups as a growth inhibitor in wheat embryos and Aegopodium tubers (Bennet-Clark and Kefford, 1953). Further research confirmed that the hormone does not promote leaf abscission albeit directly but through inducing the expression of the hormone ethylene. Since then, ABA has been demonstrated to be important in various plant processes e.g., seed dormancy, embryo maturation, seed germination, and responses to different environmental stresses such as drought, salinity, cold, and most recently pathogen responses (Cutler et al., 2010), (Wasilewska et al., 2008). ABA is a sesquiterpenoid (C_{15}H_{20}O_{4}) derived from a 5-carbon precursor isopentenyl and the first molecule in its synthesis is a C40 carotenoid (Cutler et al., 2010), (Nambara and Marion-Poll, 2005). ABA can exist in two isomers either 2- cis, 4- trans ABA which is biologically active, or 2- trans, 4- trans-ABA which is inactive, and the reversible isomerization takes place between the two in presence of UV light (Cutler et al., 2010).
1.6.1 ABA biosynthesis

ABA is synthesized by cleavage of C40 carotenoids from the plastidal 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway unlike similar sesquiterpenes that are derived from farnesyl diphosphate (FDP) (Nambara and Marion-Poll, 2005), (Vishwakarma et al., 2017). The original molecule in the ABA synthesis pathway is Zeaxanthin, an oxygenated carotenoid, formed in a reaction catalyzed by β-carotene hydroxylases (Finkelstein, 2013). Zeaxanthinepoxidase (ZEP) enzyme is the first enzyme in the biosynthesis pathway which catalyzes the conversion of zeaxanthin to violaxanthin. Violaxanthin can be converted to another C40 molecule neoxanthin and together trans-neoxanthin and trans-violaxanthin are isomerized into their cis-isomers i.e., cis-violaxanthin and cis-neoxanthin. The cis-isomers are then deoxygenated to 15C-xanthoxin by 9-cis-epoxycarotenoid dioxygenase (NCED) (Vishwakarma et al., 2017), (Finkelstein, 2013), (Nambara and Marion-Poll, 2005). This reaction is the first committed step in ABA-synthesis and therefore the expression of the NCED enzyme is tightly regulated (Finkelstein, 2013). Reactions up to the formation of 15C-xanthoxin take place in the plastids while the rest in the cytoplasm. Xanthoxin is found in the cytoplasm and subsequent steps occur in the cytoplasm (Wasilewska et al., 2008). cis-xanthoxin is further oxidized in two-step reactions to form ABA via an intermediate abscisic aldehyde. First, cis-xanthoxin is oxidized by dehydrogenase/reductase-like (SDR1) enzyme to abscisic aldehyde which is further oxidized by abscisic aldehyde oxidase (AAO) to form ABA. AAO requires molybdenum cofactor (MoCo) for its catalytic activity (Nambara and Marion-Poll, 2005).

1.6.2 ABA catabolism

ABA is catabolized either in hydrolysis or conjugation reactions. In hydrolysis reactions, catalyzed by a cytochrome P450 monooxygenase encoded by CYP707A gene, phaseic acid and
dihydrophaseic acid (DPA) are formed (Kitahata et al., 2005), (Nambara and Marion-Poll, 2005). Phaseic acid is biologically active although the activity rate is reduced in comparison to ABA while DPA is inactive in most bioassays. The conjugation reaction occurs on the carboxyl or hydroxyl groups in ABA where glucose is conjugated by enzyme ABA glucosyltransferase encoded by the AOG gene to form ABA-glucosyl ester (ABA-GE). ABA-GE is an inactive form of ABA and accumulates in vacuoles. Recent studies also show that ABA-GE is a form of long-distance transport of ABA (Hartung et al., 2002), (Nambara and Marion-Poll, 2005).

### 1.6.3 The roles of ABA in plant drought stress response

The ABA stress response best studied is its regulation of stomatal closure. In response to drought, ABA promotes stomatal closure to reduce water loss from the plant by transpiration. This response is mediated by Ca$^{2+}$ influx that activates both slow-activating sustained and rapid transient anion channels which create an efflux of anions such as chlorides. The anion efflux depolarizes the membrane leading to the activation of K$^+$ outward channels and the inhibition of K$^+$ inward channels leading to water efflux from the guard cell and subsequent stomatal closure due to loss of turgor pressure. Regulation of these channels by ABA is via phosphorylation by SnRK2 or CDPKs (MacRobbie et al., 1992), (Coello et al., 2011), (Wasilewska et al., 2008). Also, ABA-mediated ROS production is involved in stomatal closure (Sah et al., 2016).

ABA is referred to as a stress hormone, but it plays other roles such as seed dormancy and regulates plant growth under non-stress conditions. ABA induces seed dormancy by inhibiting embryo growth during the late embryogenesis which counteracts the action of GA which promotes seed germination (Finkelstein et al., 2002). This prevents vivipary where seeds germinate while still attached to the maternal plant. Seed germination is therefore preceded by a reduction of ABA concentrations in the embryo which results in an increase in ABA catabolites phaseic acid and
dihydrophaseic acid (Nambara and Marion-Poll, 2005), (Sah et al., 2016). On imbibition of water, the concentrations of ABA reduce therefore promoting seed germination. The seed dormancy stage enables the seed to survive stress (Finkelstein, 2013).

ABA also modulates root architecture in response to enhanced water uptake during drought (Coello et al., 2011), (Wasilewska et al., 2008). Stress responses related to ABA include increased root to shoot ratio to enhance water absorption in drought stress (Finkelstein, 2013). ABA also plays a role in flowering, pathogen responses, senescence (Finkelstein, 2013), (Wasilewska et al., 2008), enhances the accumulation of osmolytes, and induces expression of stress genes that have an ABRE cis-element (Finkelstein, 2013), (Vishwakarma et al., 2017), (Wasilewska et al., 2008).

1.6.4 ABA signaling pathway

The ABA signaling pathway has been described in detail using various approaches from forward genetics, protein crystallization, and other biochemical approaches (Giraudat et al., 1992), (Melcher et al., 2009), (Boudsocq et al., 2007). It consists of receptors, phosphatases, kinases, and transcription factors that bind ABA-induced genes in response to the ABA. ABA first binds receptors named pyrabactin resistance/pyr1-like/ regulatory components of aba receptors (PYR/PYL/RCAR) which belong to the StAR-related lipid transfer (START) superfamily (Park et al., 2009), (Melcher et al., 2009), (Santiago et al., 2009). These receptors have a ligand-binding pocket where ABA binds creating conformational changes to the receptor. The changes allow binding of PP2Cs, named aba insensitive1/2 (ABI1/ABI2), hypersensitive to ABA1/2 (HAB1/2), ABA-hypersensitive germination 1/3 (AHG1/3), and highly ABA-induced PP2C 1/2/3 (HAI1/2/3) forming a receptor-ABA-PP2C complex. PP2Cs interact with SnRK2 kinases in absence of ABA to dephosphorylate SnRK2. The dephosphorylated SnRK2 is inactive with respect to its kinase activity (Soon et al., 2012). Therefore, the protein phosphatases are negative regulators of ABA
response while the SnRK2 kinases are positive regulators (Yin et al., 2009), (Nishimura et al., 2009), (Santiago et al., 2009), (Soon et al., 2012), (Fujita et al., 2009).

Three PP2C phosphatases, ABI1, HAB1, PP2CA, and three SnRK2 kinases, SnRK2.2, SnRK2.3, SnRK2.6 have been characterized as core components of the ABA-dependent signaling pathway. A triple gene knockout mutant abi1/hab1/ppc2a was shown to have constitutive activation of kinases of SnRK2.2/2.3/2.6 (Rubio et al., 2009), on the other hand, a triple gene knockout mutant snrk2.2/3/6 was shown to be ABA-insensitive, had reduced seed dormancy, and exhibited weak responses to drought (Fujita et al., 2009). In presence of ABA, the PP2Cs are in complex with ABA-bound receptors PYRs, releasing the SnRK2 kinases from PP2C. The released free SnRK2 kinases auto-phosphorylate themselves (Ng et al., 2014) or as reported recently are phosphorylated by MAPKK-kinases (Takahashi et al., 2020). The active SnRK2 kinases in turn phosphorylate ABRE-binding factors (ABFs) which are transcription factors that bind ABA-responsive elements (ABREs) on ABA-induced genes (Furihata et al., 2006), (Johnson et al., 2002). The phosphorylated SnRK2 kinases also phosphorylate slow anion channels in the guard cells leading to stomatal closure (Lee et al., 2009). Four ABFs, ABF1/2/3/4 have been characterized as core components of ABRE binding factors. A quadruple gene knockout mutant areb1 areb2 abf3 abf1-2 was shown to have reduced expression of genes that are driven by the ABRE (Yoshida et al., 2015). All the components described above and illustrated below (Fig. 1.1) have been shown to be enough to create ABA responses by in vitro reconstitution in Arabidopsis protoplasts (Fujii et al., 2009).
Figure 1.1. A schematic diagram showing minimal core components important in the transmission of the ABA signal leading to the activation of the ABRE promoter and subsequent ABA-induced gene expression.
1.7 Phosphatidic acid (PA) a drought signaling molecule

Phosphatidic acid (PA) is a membrane phospholipid produced by the action of enzymes lysophosphatidic acid acyltransferases (LPAAT), DAG kinases (DGKs), and phospholipases. PA is an anionic phospholipid with a small head group (Fig. 1.2) in comparison to other phospholipids such as phosphatidylycholine and hence leads to the formation of curvatures on membranes facilitating vesicle fusion and fission. LPAAT enzymes are important in the last step of de novo synthesis of PA by acylation of 1-acyl-glycerol-3-phosphate in the endoplasmic reticulum and plastids (Tei and Baskin, 2020), (Munnik, 2001). Transiently, PA is produced by the action of phospholipases C or D (Fig. 1.2), which are classified depending on the point of cleavage on substrate phospholipid. Phospholipases Cs (PLCs) are either classified as either phosphoinositide PLCs (PI-PLCs) which cleave phosphatidylinositol 4,5 bisphosphate (PIP2) to form signaling molecules inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG) or as non-specific PLCs (NPCs) which hydrolyze other phospholipids (Munnik, 2001), (McLoughlin and Testerink, 2013). DAG is rapidly phosphorylated on its formation to form phosphatidic acid by the enzyme diacylglycerol kinase (DGK) (McLoughlin and Testerink, 2013). Phospholipases Ds (PLDs) on the other hand catalyze a reaction where the head groups from different phospholipids are cleaved (Munnik, 2001).

The different phospholipases and their different intercellular locations create diverse signaling pathways involving varied species of PA (Rodas-Junco et al., 2020). There are 9 *PI-PLC* and 6 *NPC* genes in *Arabidopsis thaliana* (Wang et al., 2020). There are 7 *DGK* genes (McLoughlin and Testerink, 2013), 12 *PLD* genes in *Arabidopsis thaliana*, three α, two β, three γ, one δ, one ε, two ζ (Testerink and Munnik, 2005). PLDα, β, γ, δ, and ε contain a Ca$^{2+}$ dependent phospholipid binding C2 domain while PLDζ1 and ζ2 have a pleckstrin homology (PH) and phox
homology (PX) domain. However, all the PLDs contain two HxKxxxD (HKD) motifs important in the catalytic activity of the enzymes (Li et al., 2009), (Wang et al., 2006). PA is hydrolyzed by lipid phosphate phosphohydrolases (LPPs) (Fig. 1.2) to form DAG or by phospholipase A (PLA) into lyso-PA (Brindley and Waggoner, 1996). The diverse number of PA forming enzymes create a large repertoire of downstream responses related to PA. In addition, the different enzymes exhibit differences in subcellular location, substrate preferences, mode of activation, and this creates spatial temporal regulation of action by PA. Different PA molecular species based on the type of acyl chains on the sn-1 and sn-2 positions further creates a diverse mode of action by PA (Li et al., 2009), (Wang et al., 2006).

1.7.1 PA as a signaling molecule

PA is produced in plants in response to both biotic and abiotic stress such as pathogens, (Andersson et al., 2006) cold, (Arisz et al., 2013) drought, wounding, salinity and in response to ABA (Zhang et al., 2004), (Katagiri et al., 2001), (Bargmann et al., 2009). It is also produced in response to ROS production (Zhang et al., 2009). PA has also been implicated in plant growth and development e.g., in seed germination and root growth (Li et al., 2009), (Testerink and Munnik, 2005). PA in normal circumstances constitutes a small portion of total lipids in a plant about 1-2% of total phospholipids in a cell (Munnik, 2001), (Li et al., 2009).
When the plant is then exposed to stress, there is a rapid synthesis of PA within minutes to induce a signal and this PA is then rapidly hydrolyzed to downregulate the signal (Li et al., 2009), (Testerink and Munnik, 2005). PA derived from the PLC/DGK pathway is involved in pathogen elicitor responses in addition to a response to cold (McLoughlin and Testerink, 2013). PA from the PLC/DGK pathway was shown to increase in tomato cells treated with elicitors N, N', N", N‴-tetraacetylchitotetraose, xylanase, and the flagellin-derived peptide flg22 (van der Luit et al., 2000) or AVR4 elicitor (Jong et al., 2004) and in Arabidopsis suspension cells due to cold (Ruelland et al., 2002). PA from the PLD pathway is also linked to pathogen responses. \( PLD_{\gamma 1} \) and \( PLD_{\beta} \) genes were induced in \textit{Arabidopsis thalami} on infiltration with \textit{Pseudomonas syringae} (Wang et al., 2006). PA produced via the PLD pathway is involved in both freezing and cold responses. \( PLD_{\alpha 1} \)
antisense Arabidopsis were found to be more tolerant to freezing while *pldδ* knockout Arabidopsis were highly susceptible to freezing stress indicating a diversified response (Wang et al., 2006).

Several studies have reported a link between ROS and PA. Exogenously applied PA enhances the production of ROS while application of H$_2$O$_2$ activates PLD and this activity is abrogated in *pldδ* knockout plants (Testerink and Munnik, 2005). PLDζ 1 is involved in root hair patterning and its partial inhibition changes the root hair pattern while the addition of PA reduced primary root growth in Arabidopsis seedlings overexpressing Glyceraldehyde-3-phosphate Dehydrogenase (GAPDH) (Wang et al., 2006), (McLoughlin and Testerink, 2013). PA is also important in microtubule organization. On addition of 1-butanol, a PLD inhibitor or in *pldα1* knockout mutant's microtubule disorganization is observed and disorganization is more severe in salt stress (Wang et al., 2006). PA is also rapidly formed during wounding due to the action of PLDα1 (Munnik, 2001), (Wang et al., 2006). In addition, PA produced in the PLC/DGK pathway also plays a role in root growth, the DGK inhibitor R59022 was found to inhibit root elongation and lateral root formation in Arabidopsis (Wang et al., 2006). The formation of PA and the downstream responses that have been previously reported are summarized below in (Fig. 1.3).
1.7.2 Role of PA in drought stress and ABA signaling

PA is a known regulator in osmotic stress responses which include drought, salinity, and in response to stress hormone ABA. PLD enzymes are activated in response to dehydration (Katagiri et al., 2001), (Munnik et al., 2000), salinity (Bargmann et al., 2009), and in response to ABA (Zhang et al., 2004). In addition, *pldα1* and *pldδ* single- and double KO mutants were found to be overly sensitive to salt stress (Bargmann et al., 2009) and less sensitive to ABA (Zhang et al., 2004). PI-PLC enzyme was also found to be activated by dehydration and salt stress but not ABA (Takahashi et al., 2001). ABA responses were also reported to be regulated by PLDs. PLDα1 has been shown to enhance ABA-mediated responses in water stress response and stomatal closure. *pldα1* mutants or PLDα1 antisense plants or application of 1-butanol, a known inhibitor of PLDα1, led to reduction of ABA-mediated stomatal closure. The addition of PA to the mutants restored the ABA response (Zhang et al., 2004), (Choudhury and Pandey, 2016), (Jacob et al.,
1999), (Ritchie and Gilroy, 1998), (Sang et al., 2001). In addition, PA binds ABI1, a negative regulator of ABA responses, thus promoting ABA signaling (Zhang et al., 2004), (Mishra et al., 2006). PLDα1 and its product PA also bind Gα (GPA1) of the heterotrimeric G-protein signaling complex, regulating ABA action in stomatal closure (Mishra et al., 2006), (Zhao and Wang, 2004).

1.8 Goals and specific objectives of dissertation research

ABA signaling is an important aspect in plant growth and development, but its temporal dynamics are poorly understood. In addition, PA has been shown to be involved in ABA signaling but its subcellular localization or its effect on the ABRE promoter activity has not elucidated. Therefore, the goals and objectives of my dissertation research are:

1. Investigate the role of PA in ABRE promoter activity.
2. Determine the spatiotemporal synthesis of PA due to action of ABA or as a response to salt stress.
3. Determine ABA signaling dynamics that regulate ABRE promoter with a function of time.
CHAPTER 2.
PHOSPHATIDIC ACID DOES NOT UP REGULATE GENE EXPRESSION OF RD29A, A MARKER OF ABA SIGNALING PATHWAY

2.1 Introduction

Abscisic acid (ABA) is an important plant hormone that regulates plant processes such as seed maturation and dormancy. ABA also mediates the response of the plant to abiotic stresses such as drought, cold, freezing, and salinity (Sondheimer et al., 1968), (Finkelstein et al., 1985), (Mantyla et al., 1995), (Borovskii et al., 2002). ABA-mediated responses to drought stress and salinity include regulation of stomatal closure (Kriedemann et al., 1972), (Steuer et al., 1988), (Jackson and Hall, 1987) and altered gene expression (Gómez et al., 1988), (Mundy and Chua, 1988). These two responses are distinct in the time of response. While stomatal regulation takes minutes, ABA-induced gene expression usually takes hours. The fast response in stomata occurs due to protein modification by phosphorylation which leads to the activation of anion channels and subsequent efflux of ions and water, out of the guard cells leading to loss of turgor and subsequent stomatal closure (Schmidt et al., 1995), (Pei et al., 1997). The slow response occurs by ABA-induced expression of genes such as RD29A, RD29B, RAB18, leading to accumulation of respective proteins important for sustained stress tolerance (Xiong et al., 2002), (Zhu, 2002).

These two responses, however, share the core components of the ABA signaling pathway which starts with the binding of ABA to pyrabactin resistance/pyr1-like/ regulatory components receptors (PYR/PYL/RCAR) (Park et al., 2009), (Melcher et al., 2009), (Santiago et al., 2009) which causes the binding and inhibition of protein phosphatases PP2Cs (Santiago et al., 2009), (Soon et al., 2012), (Fig. 2.1). In the absence of ABA, PP2C binds and dephosphorylates SNF1-related protein kinase 2 (SnRK2) (Umezawa et al., 2009), (Yoshida et al., 2010). Therefore, in the presence of ABA, SnRK2 kinases are free and activated ready to phosphorylate.
In stomatal closure, the phosphorylated SnRK2.6/OST1 (open stomata 1) phosphorylates the S-type anion channels SLAC1 activating them (Geiger et al., 2009), (Lee et al., 2009), (Fig. 2.1). This leads to anion efflux from the guard cells causing membrane depolarization and subsequent activation of K⁺ efflux channels. This causes water efflux leading to a loss of turgor and eventual closure of the stomata (Schroeder et al., 1984). In gene expression, the SnRK2.6 kinase and additional kinases SnRK2.2 and SnRK2.3 phosphorylate ABRE-binding factors (ABFs) which are transcription factors that bind ABA-responsive elements (ABREs) on ABA-induced genes (Johnson et al., 2002), (Furihata et al., 2006).

The involvement of phosphatidic acid (PA) in the core ABA signaling pathway with respect to stomatal closure has been demonstrated. PA is a membrane component and transiently formed by enzymes phospholipase Ds, within the first 10 minutes after plants are exposed to ABA (Zhang et al., 2004), (Ritchie & Gilroy, 1998). PA then binds ABA insensitive 1 (ABI1), a PP2C phosphatase, inhibiting its phosphatase activity and thought to tether it to the plasma membrane specifically, which makes the SnRK2 freely mobilized within the cells to activate the SLAC1 channel in stomatal closure (Ritchie and Gilroy, 1998), (Mishra et al., 2006), (Fig. 2.2). ABA-induced stomatal closure was inhibited in single mutant plda1 and double knockout plda1/δ while the addition of PA induced stomatal closure and inhibited stomatal opening (Uraji et al., 2012), (Jacob et al., 1999), (Mishra et al., 2006). The formation of PA also stimulates ABA-induced ROS production by binding and activating NADPH oxidase RbohD/F in guard cells to promote stomatal closure (Zhang et al., 2009). Further regulation involves PLDα1 protein which was shown to bind the heterotrimeric G protein subunit Ga limiting ABA-mediated inhibition of stomatal opening (Zhao & Wang, 2004).
However, the involvement of PA formed by the PLDs has not been clearly demonstrated in downstream ABA-mediated gene expression, despite sharing the core components of the ABA signaling pathway with the ABA-regulated stomatal closure. The study of the effect of PA on downstream ABA-mediated gene expression has been investigated using knockout mutants and over-expression of the PLD genes. *PLDa1* gene overexpression led to dehydration enhanced expression of both *RD29A* and *RAB18* and addition of PA on aleurone protoplasts enhanced ABA-induced gene expression of *RAB18* (Peng et al., 2010), (Ritchie & Gilroy, 1998). On the other hand, a study on *pldδ* gene knockout mutant found that ABA upregulates expression levels of *RD29A* and *RAB18* genes (Distéfano et al., 2015).

![ABA Signaling Pathway Cartoon](image)

**Figure 2.1.** A cartoon showing the ABA signaling pathway leading to both the rapid stomatal closure response and slow ABA-induced gene expression. The pathway includes PA and its role in regulating the ABA-signaling pathway as has been described in stomatal closure assays.

In another study, the addition of the chemical inhibitor for PLD, iso-butanol, was found to have no effect on sodium chloride mediated induction on the gene expression of *RD29A*, while it up-regulates the gene expression of *RAB18* (Thiery et al., 2004). Another discrepancy is the subcellular localization of ABI1 and PLDα1 protein. As it was described above, PA formed by
PLDα1 was shown to bind ABI1 and tether it to the plasma membrane, therefore, inhibiting the ABA signaling pathway (Zhang et al., 2004), (Fig. 2.2). However, recent studies have shown that ABI1 localizes, in addition to the plasma membrane, to both the cytosol and the nucleus. The nucleic localization was determined to be important to enable its inhibitory role of ABA-induced gene expression (Umezawa et al., 2009), (Moes et al., 2008), (Li et al., 2012). Subcellular localization of the PLDα1 was also found in the plasma membrane, microtubules, and cytosol (Li et al., 2009). For our study, we wish to investigate the reasons for these contradicted findings, namely:

1. Whether PA regulates the *RD29A* promoter. Why do both knockout and overexpression of a PLD gene upregulate the *RD29A* gene expression?

2. Subcellular localization of PA that inhibits ABI1. Does it occur only in the plasma membrane or also in the internal membranes?

![Figure 2.2](image)

Figure 2.2. A cartoon showing the role of PA in the ABA signaling pathway as proposed in (Zhang et al., 2004). In absence of ABA, ABI1 a PP2C binds SnRK2 kinases inhibiting them (a). PA formed on the plasma membrane due to action of ABA (b) tethers ABI1 and this leaves the SnRK2 kinases free and activated to phosphorylate downstream signaling components like SLAC1 and ABF.

Because the promoter of the *RD29A* gene contains the *ABRE* cis element (Yamaguchi-Shinozaki and Shinozaki, 1994) expression of the *RD29A* gene has been most widely used as a marker for the activation of core components of the ABA signaling pathway in the past.
(Nakashima et al., 2006), (Zhu et al., 2017). The expression is determined by detecting the accumulated mRNA in cells (Lee et al., 2016), (Narusaka et al., 2003) or by detecting the activity of a recombinant protein, such as luciferase (LUC), fluorescent protein (FP), or β-glucuronidase (GUS), expressed under an artificial RD29A promoter (Ishitani et al., 1997), (Yamaguchi-Shinozaki and Shinozaki, 1993), (Msanne et al., 2011). To this end, we analyzed the effect of PA formed by PLD on the RD29A promoter activity to investigate the reasons for the two contradicted findings mentioned above.

2.2 Materials and methods

2.2.1 Plant growth conditions

Arabidopsis thaliana seeds were sterilized in 70% Ethanol for 1 minute then in 50% bleach, 0.05% triton for 10 minutes. The seeds were then rinsed 6 times with sterile distilled water, and then plated on 0.8% agar ½ MS strength. They were then stratified at 4°C for 3 days, then grown in a growth chamber under a growth cycle of 16 hours light 8 hours dark with light set at 100 µmol m⁻² s⁻¹ and at 22°C.

2.2.2 Luminescence assay with transgenic Arabidopsis thaliana

Twenty-five-day old transgenic Arabidopsis thaliana plants (CS67900) (RD29A::LUC) on an agar plate were sprayed with 200 µM ABA and with the same volume of DMSO as a control for different time periods (0, 5, 8, 12, and 24 hours). Seedlings were then harvested one hour before the start of the dark cycle and frozen immediately in liquid N₂ and kept at –80°C until analysis. For analysis, frozen seedlings were ground using a mortar and pestle to form a powder. The powder was mixed with passive lysis buffer (Promega cat# E1941), the mix was then vortexed and centrifuged at 14,000 rpm for 10 minutes. 40 µL of the supernatant was placed in a well on a 96 well plate (Thermo Fischer cat# 267350) and mixed with 100 µL luciferase assay substrate
(Promega cat. #E151A). Relative Luminescence Unit (RLU) was detected using a Veritas TM microplate luminometer. 3 RLU readings were made for each well.

2.2.3 Eight-hour RD29A::LUC expression assay

One-week old seedlings with RD29A::LUC (Col) (CS67900) or CAMV35S::LUC (Col) (CS25237) and (CS25230) or pld a1/RD29A::LUC or pld δ/RD29A::LUC were placed in wells of a 96-well plate (Thermo Fischer cat# 267350) and incubated with either 200 µM ABA only or 200 µM ABA with 0.6 % iso-butanol or 0.6% tert-butanol or 0.6% sec-butanol or 1 µM FIPI (Millipore Sigma cat# 528245) or with 100 µM Egg yolk PA (Avanti Polar lipids cat# 840101). As a control, equal amounts of DMSO were used. Three seedlings were placed in one well for RD29A::LUC and one seedling for CAMV35S::LUC and each treatment replicate had a total of six wells, in total eighteen or six seedlings. Continuous RLU (Relative luminescent unit) from time zero (incubation time) up to 8 hours was detected using 1 mM of D-luciferin (ThermoFisher cat #88293) as the substrate and a Veritas™ microplate luminometer. 3 RLU readings were made each hour.

2.2.4 Crossing of RD29A::LUC (Col) and pld a1 and pld δ mutants and luciferase expression

Knockout Arabidopsis thaliana seeds for pld a1 (Col)-SALK_067533C and pld δ (Col) - SALK_023247C were obtained from ABRC. Homozygosity of the mutant plants was confirmed using the following primers: LP CCAAAAGAGTTGTCGCTGAAG and RP CATTCTCTCACCACGTCATTG for pld a1 and LP ATCCTACAGTGCAAATCGTGC and RP AGGAAAGGAAGTCAGGTGAGG for pld δ. Homozygous RD29A::LUC and homozygous mutant seeds were germinated. On flowering, homozygous RD29A::LUC (Col) plant flowers were chosen as the male parent and the mutant plant flowers as the female parents. All stamens were removed from selected flowers on the female plant and an open flower from the male plant was used to dust pollen on the stigma of the female plant. Flowers on the female plant were allowed to
mature into a silique and seeds harvested after drying. F1 progeny was tested for luciferase activity using luciferin. F1 plants were self-crossed and the F2 progeny were confirmed to be homozygous for the mutation or homozygous for the wild type gene PLD α1 or PLD δ. The luciferase gene was determined to be present using primers: FP ACCAGGGATTTCAGTCGATGT and RP GTCCACAAACACAACACTCCTCC. F2 progeny plants were self-crossed and F3 seeds were obtained. The presence of the luciferase gene was determined from this to confirm homozygosity of the luciferase gene since homozygous plants should all breed true while heterozygous plants will have offspring with the luciferase gene at a ratio of 3:1. Seven-day-old seedlings homozygous for the mutation or wild type for the gene and homozygous for the luciferase gene were placed in wells in a 96 well plate containing ½ MS with 200 µM ABA and D-luciferin or ½ MS with DMSO and D-luciferin. RLU was detected using a Veritas™ microplate luminometer over a period of 8 hours.

2.2.5 RNA extraction for quantitative PCR

One-week-old Arabidopsis thaliana WT (Col) seedlings were treated with and without 200 µM ABA and with 200 µM ABA + 1 µM FIPI and for control, DMSO only. One-week old Arabidopsis thaliana WT (Col) or pld α1 (Col)-SALK_067533C or pld δ (Col) -SALK_023247C or pld α1/δ were treated with 200 µM of ABA and for control DMSO only. 100 mg of seedlings were collected after incubation for 4 hours and frozen in liquid nitrogen. To extract RNA, frozen seedlings were ground briefly and 1 mL of TRIzol (Ambion Life technologies cat#15596026) was added to each sample. The samples were incubated at room temperature for 5 minutes and 200 µL of chloroform was added, and the samples were mixed. Tubes were left to stand for 3 minutes at room temperature and then centrifuged at 14,000 rpm for 15 minutes at room temperature. The aqueous phase was then collected and 250 µL of isopropanol and 250 µL of a high salt solution
(0.8 M sodium citrate and 1.2 M sodium chloride) were added. The samples were again incubated at room temperature for 10 minutes and then centrifuged at 14,000rpm for 10 minutes at room temperature. After the centrifuge step, the supernatant was discarded, and 1 mL of 70% ethanol was added to clean the pellet. The mixture was mixed by flicking the tube and then centrifuged at 7,500 rpm for 5 minutes at room temperature. The pellet was air dried for 1 hour at room temperature and Kim wipes were used to collect excess ethanol from the tube. 100 µL of nuclease free water was used to dissolve the RNA and RNA concentration determined using a nanodrop spectrophotometer. RNA quality was also determined on a 2% agarose gel. *pld α1/δ* double knockout mutation was confirmed using the following primers: for *α1* mutation: *FP* ATTAAGTGCAGGGCATTGATG and *RP* CAAGGCTGCAAAGTTTCTCTG and for *δ* mutation: *FP* ATCCTACAGTGCAAATCGTGC and *RP* AGGAAAGGAAGTCAGGTGAGG (Xia et al., 2020).

### 2.2.6 Semi quantitative PCR

1 µg/µL of RNA was treated with 1 µL DNAse (Promega cat# M6101) at 37°C for 30 minutes. 1 µL of DNAse stop was added to stop the reaction and the mix was incubated at 65°C for 10 minutes. The treated RNA was then used to synthesize first-strand cDNA using (iScript cDNA synthesis kit Biorad cat# 1708890). 1 µL of iScript reverse transcriptase was added to 1 µg/µL treated RNA together with the reaction mix and 2 µL of 10 mM dNTP mix (NEB cat# N0447S) and adjusted to a 20 µL volume using nuclease-free water. Reaction settings for the synthesis were: 5 minutes at 25°C, 20 minutes at 46°C, and 1 minute at 95°C. For amplification, the first strand sample was diluted by 1/2 and 0.125 µL NEB Taq DNA polymerase (cat # M0273S) was added together with the 0.5 µL of 12 µM LUC and ACTIN forward and reverse primers each per reaction, 0.5 µL 10mM dNTP mix and 5 µL reaction mix. The amplification settings were: 2 minutes 96°C,
1-minute 94°C, 1-minute 64°C, 1-minute 72°C, and the cycles were repeated from step 2. A total of 28 cycles were run. The products were separated on 2% agarose gel with ethidium bromide and a Biorad gel analyzer chemidoc XRS+SYS was used to detect and image the bands using UV light illumination. ImageJ (Fiji) rectangle tool was used to determine the intensities of the bands. Primers used where *ACTIN* was the reference gene were FP CCCGCTATGTATGTCGC and RP AAGGTCAGACGGAGGAT and for *LUC* the primers were FP GTTGTGGTTTGAGCAGCAGAAAGAC and RP CAACTCCTCCGCGCAACTTTTTTCG.

2.2.7 quantitative PCR

RNA was treated with DNase as above and this was used in a 1-step qPCR reaction using iTaq Universal SYBR green one-step kit cat# 172-5150 to quantify *RD29A* native gene expression. 500 ng/µL of treated RNA was mixed with 5 µL of the SYBR green reaction mix, 0.125 µL iScript reverse transcriptase, and 0.25 µL of 12 µM of both forward and reverse primers. The thermal cycling settings were 10 minutes at 50°C, 1 minute at 95°C, 15 seconds at 95°C, 60 seconds at 60°C for 40 cycles. Data were analyzed using Quant Studio Real-Time PCR Software from Applied biosystems. Primers used for *ACTIN* as reference gene were FP CCCGCTATGTATGTCGC, RP AAGGTCAGACGGAGGAT, and *RD29A* native gene FP CACTCAACACACACCGAGCAG and RP GGTGCATCGATCACTTCAG.

2.2.8 Stomatal closure assay

Four- to six-week-old *Arabidopsis thaliana* WT (Col) or *pld α1* or *pld δ* leaves were harvested and placed in a stomatal opening solution containing 5mM KCl, 50 µM CaCl2, and 10 mM MES-Tris, pH 6.15 (Uraji et al., 2012) for 2 hours to induce stomatal opening. The leaves were then incubated with 100 µM of ABA only or 100 µM ABA with 0.6% butanol or 0.1 µM FIPI or 1 µM FIPI, and 100 µM PA only for 2 hours. The leaves were then shredded in a mini blender for 20
seconds. The mix was then filtered using BD Falcon cell strainer 100 µm (cat#352360) and the epidermal layer pieces were placed on a Corning glass slide (cat#2948). 100 µL of the opening stomata solution was added to the pieces and covered with a Corning coverslip (cat# 2980). Stomata images were observed and captured using a Leica DMI microscope using a dry 40X objective lens. The stomata images were then analyzed using imageJ (Fiji) line tool and stomatal aperture was determined as the ratio between the width and length of the stomata.

2.2.9 Thin Layer Chromatography assay

Twenty Arabidopsis thaliana WT (Col), pld α1, or pld δ seedlings were incubated with NBD-PC (16:0-06:0) (Avanti polar lipids cat#810130) for 21/2 hours in the dark at room temperature. Twenty seedlings of pldα1/δ double knockout mutant were incubated with NBD-PC (16:0-06:0) (Avanti polar lipids cat#810130) for 2 hours in the dark at room temperature. The seedlings were then incubated with 200µM ABA or same volume of DMSO as control for 30 minutes. The seedlings from all the samples were then collected and weighed for lipid extraction. The seedlings were placed in 400 µL of hot isopropanol with 0.01% BHT and incubated for 15 minutes at 75°C. 1.2 mL of extraction mixture containing chloroform/methanol/water (30:41.5:3.5) was added to the seedling/isopropanol mix and samples were left on a shaker at 100 rpm for 24 hours. The solvent was then collected, and seedlings discarded. The solvent was then dried at 70°C and under a stream of nitrogen and the extract dissolved in 40 µL of chloroform. 10 µL of the extract was loaded onto a silica-gel TLC plate with a binder Polymeric fluorescent indicator from (Sigma-Aldrich cat# Z193291) and lipids separated using chloroform/ethanol/water/trimethylamine (30:35:7:35) solvent mix.
2.2.10 Microscope assay

One-week old *Arabidopsis thaliana* WT (Col) seedlings of *pldα1/δ* knockout mutants were incubated with NBD-PC or NBD-PA on a Corning glass slide (cat#2948) and covered with Corning cover slip (cat# 2980). Images were taken on Olympus SpinSR10 microscope using a 40x/1.4 oil objective lens. Images were taken in a z-stack of 30 µm thickness with a z-step size of 1µm. Fluorescence was detected using a 464 nm excitation and 531 nm emission. Obtained images were then projected in z-axis using ImageJ (Fiji) software.

2.3 Results

2.3.1 PLD inhibitor iso-butanol abrogates *RD29A::LUC* activity

To determine whether PA affects gene expression through the core components of the ABA signaling pathway, we first tested the transgenic *RD29A::LUC* plants. The transgenic plants have an *RD29A* promoter fused to a firefly luciferase gene that can be detected for the determination of the promoter activity (Ishitani et al., 1997). Treatment of the plants with 0.6 % iso-butanol to inhibit PLD activity in plants has been widely conducted in the past (Motes et al., 2005), (Thiery et al., 2004), (Johansson et al., 2014). Hence, we treated the transgenic plants with 200 µM ABA or 200 µM ABA + 0.6% iso-butanol, and luciferase expression was used to determine the activation of the *RD29A* promoter. When the plants were exposed to ABA, luciferase activity was detected by the emission of luminescence increase (Fig. 2.3a). However, the expression was abrogated when the plants were exposed to ABA with 0.6% iso-butanol (Fig. 2.3b). For control, a transgenic plant *CAMV35S::LUC* was used. The transgenic plants *CAMV35S::LUC*, have a *CAMV35S* promoter fused to a firefly luciferase gene. The promoter does not contain *ABRE* cis-element and therefore the plants act as a control for the luciferase reaction. The *CAMV35S::LUC* transgenic plants showed minimal change in luciferase expression by ABA or iso-butanol (Fig.
2.3a & b). Hence, we concluded that the reduction in luminescence was due to the suppression of the \textit{RD29A::LUC} promoter activity but not the suppression of luciferase enzymatic activity by butanol. Additional control experiments using tert-butanol and sec-butanol with ABA were also conducted since they do not inhibit the PLD enzymatic activity (Johansson et al., 2014), (Jia and Li, 2018), (Distéfano et al., 2008), confirming again that the reduction in luminescence is due to specific inhibition on PLD by iso-butanol but not suppression of luciferase enzymatic activity by butanol (Fig. 2.3c & d). From these results, we hypothesized that PA affects the core components in the ABA signaling pathway to upregulate ABA-related gene expression.

Figure 2.3. Iso-butanol abrogates luciferase expression from the \textit{RD29A} promoter but not from the \textit{35S} promoter. Normalized relative luminescence intensities of \textit{RD29A::LUC} and \textit{CAMV35S::LUC} seedlings treated with ABA only in (a) or with ABA + 0.6 % butanol in (b). The \textit{RD29A::LUC} seedlings were also treated with ABA + 0.6% tert-butanol (c) and with ABA + 0.6% sec-butanol (d). Relative luminescence intensities were normalized against the control samples that were treated with DMSO. Each data point represents means of 3 replicates with error bars representing standard error from the mean.
2.3.2 PLD knockout mutants plda1, pldδ, and plda1/δ do not show abrogated ABA-induced RD29A gene expression

To determine which PLDs are involved in the regulation of gene expression in the ABA signaling pathway, we obtained plda1, pldδ, and plda1/δ gene knockout mutants. These mutants have been shown previously to have impaired ABA-mediated signaling in stomatal closure (Uraji et al., 2012). First, we analyzed whether these mutants are impaired in overall PLD activities in plants as previously reported (Zhang et al., 2004), (Ritchie and Gilroy, 1998) i.e., abrogated synthesis of PA and reduced sensitivity to ABA-mediated stomatal closure. We analyzed the PLD activity in the plants by treating them with fluorescently labeled PC (NBD-PC) and tracked its conversion to PA (NBD-PA) with thin layer chromatography (TLC) (Fig. 2.4 & 2.5). The mutants showed reduced PA synthesis even without ABA treatment (Fig. 2.5). The plda1 mutant had reduced PA synthesis than the WT although the activity seemed not to be significantly lower than the WT. The pldδ mutant had significantly reduced PA synthesis in comparison to the WT (Fig. 2.5). PA synthesis in the double knockout mutant of plda1/δ was especially low even when it was treated with ABA (Fig. 2.4 & 2.5). These results confirmed that the knockout mutants, especially the plda1/δ double knockout, have reduced PLD activity in the plants.

Figure 2.4. Determination of PLD activity in plants. A TLC analysis was conducted with lipid extracts from WT and plda1/δ double knockout mutant plants. The plants were incubated with fluorescently labeled phospholipids PC (NBD-PC) for 2 hours, and then with or without ABA for 30 min.
Figure 2.5. The plda1, plδ, and plda1/δ mutant plants are impaired in PA synthesis from PC. PA synthesis was quantified in WT, and plda1, plδ and plda1/δ mutants using fluorescently labeled lipid, NBD-PC. The PLD activity was determined as a ratio of NBD-PA/ (NBD-PA + NBD-PC). The plda1/δ knockout plants were incubated with and without ABA. The bars show means from three replicates of 20 seedlings each and the error bars represent standard error to the mean. One-way means ANOVA was conducted to determine significant differences among the samples. Letters denote statistical differences with a Compare means Student’s t-test (p<0.05).

Figure 2.6. Mutant plants show reduced sensitivity to ABA in stomatal closure. Stomatal aperture sizes of WT, and plda1, plδ and plda1/δ mutants when treated with 100 µM ABA or DMSO. Each bar except for plda1/δ represents average stomatal size from 3 replicates of 60 stomata assayed with error bars representing standard error to the mean. For plda1/δ, the data is from one replicate of 20 stomata assayed and respective error bars representing standard error to the mean. p values from a Student’s t-test between samples treated with and without ABA are shown.

We also examined whether the mutants were impaired in stomatal closure as previously reported. Mutant and WT plants were treated with ABA or DMSO and the sizes of the stomatal
apertures were compared (Fig. 2.6). We found out that the sizes of stomatal apertures in \textit{plda1} and \textit{plda1/δ} mutants were not significantly different with and without ABA treatment. This result confirmed the previous reports that the \textit{plda1} and \textit{plda1/δ} plants have reduced sensitivity against ABA in stomatal closure (Uraji et al., 2012).

We then generated \textit{RD29A::LUC} transgenic plants that lack the \textit{PLDa1} or \textit{PLDδ} gene to analyze the effect of the mutant on the \textit{RD29A} promoter activity. Namely, the \textit{plda1} and \textit{pldδ} mutants were crossed with \textit{RD29A::LUC} transgenic plants to obtain \textit{plda1/RD29A::LUC} and \textit{pldδ/RD29A::LUC} plants. Plants that were WT for the \textit{PLDa1} and \textit{PLDδ} gene and carry \textit{RD29A::LUC} were also obtained from offspring of the crosses (\textit{plda1 x RD29A::LUC} and \textit{pldδ x RD29A::LUC}, respectively) to act as control plants (\textit{WT/ RD29A::LUC}). The successful crossing of the plants was confirmed using PCR with respective primers to identify the presence of a T-DNA insertion in each gene, \textit{PLDa1}, and \textit{PLDδ}, respectively (Fig. 2.7 & 2.8). The \textit{plda1/δ} double knockout was also profiled for the mutations using the respective primers (Fig.2.9).

Figure 2.7. Knockout mutants \textit{plda1} were crossed with \textit{RD29A::LUC} to generate \textit{plda1/RD29A::LUC} (a) and \textit{PLDa1/RD29A::LUC} (b). Results of PCR reactions with primers to detect WT gene (\textit{PLDa1}) or primers to detect T-DNA insertion in the \textit{PLDa1} gene are shown on the left. On the right are PCR results showing reactions with primers to detect the luciferase expression cassette (\textit{RD29A::LUC}). Nine independent plants from established lines were used to confirm the homozygous status of the luciferase expression cassette.
Figure 2.8. Knockout mutants *pldδ* were crossed with *RD29A::LUC* to generate *pldδ/RD29A::LUC* (a) and *PLDδ/RD29A::LUC* (b). Results of PCR reactions with primers to detect WT gene (*PLDδ*) or primers to detect T-DNA insertion in the *PLDδ* gene are shown on the left. On the right are PCR results showing reactions with primers to detect the luciferase expression cassette (*RD29A::LUC*). Nine independent plants from established lines were used to confirm the homozygous status of the luciferase expression cassette.

Figure 2.9. Knockout mutant *pldα1/δ* was confirmed to be a double knockout mutant using PCR reactions with primers to detect WT genes (*PLDα1* and *PLDδ*) or primers to detect T-DNA insertion in the *PLDα1* and *PLDδ* genes.

To investigate how the mutants affect the activity of the *RD29A* promoter, the kinetics of luciferase activity were determined in *pldα1/RD29A::LUC* and *pldδ/RD29A::LUC* plants. The *pldα1/RD29A::LUC* mutants showed significantly increased luciferase activity (Fig. 2.10a) in comparison to the *PLDα1/RD29A::LUC* plants between 2- and 4-hour time points while the *pldδ/RD29A::LUC* did not show any differences with the *PLDδ/RD29A::LUC* (Fig. 2.10b).
Figure 2.10. Mutant plants show enhanced or similar luciferase expression from the *RD29A* promoter. The *pldα1/RD29A::LUC* plants have significantly higher luciferase activity in comparison to *PLDα1/RD29A::LUC* plants (a) while the *pldδ/RD29A::LUC* plants do not show any significant differences in comparison to *PLDδ/RD29A::LUC* plants (b). Luminescence intensities at each time point with ABA were normalized by those with DMSO. The graphs show an average of 3 independent experiments in which luminescence from 18 seedlings was measured. Error bars represent standard error to the mean. A Student’s t-test was conducted on data in (a) and luciferase expression at time points between 2 and 4 hours was found to be significantly different (*p*<0.01). The bar denotes the time points where the expression was significant.

This result using the mutants was contradictory to the result obtained with iso-butanol in which luciferase activity was significantly abrogated by inhibiting PLD activity. To understand the cause of the contradicted result, we analyzed the expression of the native *RD29A* gene in the mutants with and without ABA by quantitative PCR (qPCR). All the mutants, *plda1*, *pldδ*, and *plda1*/*pldδ* showed increased expression levels of *RD29A* in comparison to WT without ABA treatment although the increase was not significant (Fig. 2.11a). On treatment with ABA, all mutants showed similar *RD29A* expression levels as the WT although the *RD29A* expression levels tended to be lower in the single mutants of *plda1* and *pldδ* (Fig. 2.11b).

However, neither of the changes were significant in the mutants in comparison to the WT plants. These results suggested that PA produced by PLD activity has a negligible effect on the *RD29A* promoter activity, and iso-butanol may downregulate the *RD29A* promoter activity independent from the status of the PLD activity.
Figure 2.11. Mutant plants show relatively similar expression of native *RD29A* gene expression with WT plants. Relative expression of *RD29A* gene in WT and mutant seedlings after incubation without (a) and with (b) ABA for 4 hours. qPCR was conducted with primers to amplify *RD29A* and *ACTIN* mRNAs. Relative expression levels of the *RD29A* gene were calculated by normalizing with *ACTIN* mRNA. The analysis found relative expression levels of the *RD29A* gene tend to be higher in all mutant plants, in comparison with WT plants without ABA although all of them are not significantly higher. When treated with ABA, no significant differences were observed between WT and the mutant plants (b). Each bar represents mean expression levels of 3 replicates with error bars representing standard error to the mean. One-way means ANOVA was conducted to determine significant differences among the samples and no significant differences (ns) were observed.

### 2.3.3. PLD inhibitor FIPI does not abrogate *RD29A::LUC* activity

To examine the hypothesis that iso-butanol downregulates the *RD29A* promoter activity independent from the status of the PLD activity, we used 5-Fluoro-2-indolyl des-chlorohalopemide (FIPI), another PLD inhibitor that is more often used in animal studies (Su et al., 2009), (Stegner et al., 2013), (Stricker et al., 2021) than plant studies (Gao et al., 2013) due to less deleterious effect, compared to iso-butanol, in animal cells (Su et al., 2009). First, we confirmed inhibition of
stomatal closure by FIPI. On addition of 0.1 µM or 1 µM FIPI, ABA-mediated stomatal closure was inhibited significantly with 1µM FIPI (Fig. 2.12). A similar result was observed when the stomata were treated with ABA and 0.6% iso-butanol. We then treated the RD29A::LUC plants with either ABA only or with ABA and 1 µM FIPI or with 1 µM FIPI only to determine the effect of luciferase expression on the RD29A::LUC plants. The addition of 1 µM FIPI to the ABA-treated plants did not alternate the stimulation of luciferase activity (Fig. 2.13a). CAMV35S::LUC plants were also treated with the same and no significant effects were observed (Fig. 2.13b). We then analyzed the accumulation of the luciferase (LUC) mRNA in the WT/RD29A::LUC transgenic plant (Fig. 2.14a) and the RD29A mRNA in WT plants (Fig. 2.14b). The results showed 1 µM FIPI does not significantly affect the accumulation of the RD29A gene and luciferase gene in WT plants and transgenic plants, respectively. This supports the idea that inhibition of PLD activity and PA do not alternate the RD29A promoter activity.

Figure 2.12. FIPI inhibits ABA-mediated stomatal closure similar to 0.6% butanol. Stomatal aperture sizes on WT leaves treated with and without ABA, ABA + 0.6% butanol, ABA + 0.1 µM FIPI, or ABA + 1 µM FIPI for 2 hours. Each bar represents the means of 3 replicates of 60 stomata assayed with error bars representing standard error to the mean. One-way means ANOVA was conducted to determine significance between samples. Letters denote statistical differences with a Compare means Student’s t-test (p<0.05).
Figure 2.13. FIPI does not alter luciferase activity in the RD29A::LUC plant. Normalized relative luminescence intensities are shown of RD29A::LUC (a) and CAMV35S::LUC (b) seedlings treated with ABA, ABA + 1 µM FIPI, or 1 µM FIPI only. Relative intensities were determined and normalized against control (with DMSO) at each time point. The graph shows results from the means of 3 replicates with error bars from standard error to the mean.

Figure 2.14. Expression and activation of RD29A is not alternated by FIPI. Plants were incubated for 4 hours with and without ABA or with ABA + 1 µM FIPI. In (a) semi-quantitative PCR was conducted with the RD29A::LUC transgenic plants to determine relative expression level of the luciferase gene against ACTIN mRNA. In (b) qPCR was conducted with WT plants to determine relative gene expression level of the RD29A gene against ACTIN mRNA. Each bar represents average expression levels of 3 replicates with error bars representing standard error to the mean. A Student’s t-test was conducted between the +ABA and +ABA +1µM FIPI samples and the p values are shown.
**2.3.4 Addition of exogenous PA does not upregulate the *RD29A::LUC* expression**

To confirm that PA does not affect ABA-mediated *RD29A* expression, we analyzed effects of exogenously added PA in plants. First, we analyzed stomatal closure. Stomatal aperture sizes significantly reduced in WT, *plda1*, and *pldδ* on the addition of PA (Uraji et al., 2012), (Fig. 2.15). However, PA does not significantly change the luciferase activity in the *RD29A::LUC* transgenic plants even together with ABA (Fig 2.16a). These results confirmed that PA does not affect the *RD29A* promoter activity.

![Figure 2.15. PA promotes stomatal closure in both WT and mutant plants. Stomatal aperture sizes of WT, and *plda1*, and *pldδ* plants were treated with and without 100 µM PA for 2 hours. Each bar represents mean stomatal sizes from 3 replicates of 60 stomata assayed with error bars from standard error to the mean. A Student’s t-test was conducted between samples treated with PA and those not treated, and the respective *p* values are shown.](image-url)
Figure 2.16. Exogenously added PA does not increase luciferase activity in the RD29A::LUC plants. Normalized relative luminescence intensities in the RD29A::LUC plant (a) and CAMV35S::LUC plant (b) are shown. Seedlings were treated with ABA only or with ABA + 100 µM PA or with 100 µM PA only. Relative intensities were determined and normalized against control (with DMSO) at each time point. The graph shows results from the means of 3 replicates with error bars from standard error to the mean.

2.3.5 Exogenously added PA is incorporated within the internal membranes within 2 minutes

We found that PA had a negligible effect on the RD29A promoter activity. We then questioned ourselves why PA affects stomatal closure but not the gene expression during ABA signaling transduction although the core components of the signaling pathway are shared between stomatal closure and gene expression. A previous report suggested that inhibition of ABI1 by PA formed by PLD activity during stomatal closure was caused by tethering of ABI1 (PP2C) on the plasma membrane (Zhang et al., 2004). Therefore, we hypothesized that the regulation of the core components in the ABA signaling pathway by PLD activity would be limited in the plasma membrane where the SLAC1 channel protein is localized to regulate stomatal closure (Hedrich, 2012). This would be the reason we did not see the effect of PA on ABA-mediated gene expression which would be mediated by internally localized ABI1 while stomatal closure would be mediated
by plasma membrane localized ABI1. With this, we analyzed PA localizations using fluorescently labeled PA (NBD-PA). The aim of the experiment was to determine whether exogenously added PA, which is first incorporated in the plasma membrane, remained in the plasma membrane, or internalized when ABA was added. We rationalized that the exogenous added PA would remain in the plasma membrane when ABA is added. We also analyzed the localization of exogenously added PC (NBD-PC) and PA (NBD-PA) in seedlings of pldα1/δ double knockout mutant. We rationalized that exogenously added PA and PC may localize in different subcellular compartments. If so, the pldα1/δ plant would show different subcellular localization of NBD signals, compared to the WT plant, due to lacking the ability to produce NBD-PA from NBD-PC within the cells.

Our fluorescence microscopy assay found that NBD-PA was rapidly incorporated into the internal membrane within 2 minutes after NBD-PA is exposed to the seedling with and without ABA (Fig. 2.17). This suggests that PA that is formed by PLDα1 or PLDδ would be rapidly internalized even when ABA was added. This also suggested that PA formed by PLD might play a role in the ABA signaling pathway internally the same as the plasma membrane localized PA. Our fluorescence microscopy also found the distribution of both NBD-PA and NBD-PC was not distinguishable between wild type and pldα1/δ double knockout mutant plants (Fig. 2.18). Localization of NBD-PA and NBD-PC is not limited to the plasma membrane but dispersed in the internal components in both wild type and pldα1/δ double knockout mutant plants. This suggests that PLDα1 and PLDδ do not largely affect the distribution of PC and PA within cells.
Figure 2.17. NBD-PA is internalized into the cells within minutes of exposure in Arabidopsis roots. NBD-PA fluorescence was detected in Arabidopsis roots of 1-week seedlings immediately after incubating with 50 μM NBD-PA with (a) and without (b) 100 μM ABA. Images were taken at different time points as shown. A single cell is marked with a dashed-yellow line for clarification. Notice NBD signals are detected in the cytosolic area within 2 min with and without ABA.
Figure 2.18. Distribution of NBD-PC and NBD-PA are indistinguishable between WT and plda1/δ mutant plants. Arabidopsis roots were incubated with 50 μM NBD-PC or 50 μM NBD-PA with and without 100 μM ABA for 30 min. A single cell is marked with a dashed-yellow line for clarification. Notice no significant difference is observed among the samples.

2.4 Discussion

Based on the result with iso-butanol, we originally thought that PA would upregulate the RD29A promoter as it upregulates stomatal closure. However, genetics and alternative chemical inhibition assay proved that the suppression of the RD29A promoter activity by iso-butanol is not due to reduction of PLD activity and PA synthesis but most likely other factors (Fig. 2.10, 2.11, 2.13, & 2.14). Some of the animal studies found that iso-butanol had deleterious effects on cells including a reduction in phosphoinositides which are important in maintaining proper Golgi membrane structure (Su et al., 2009). Therefore, the inhibitory activity of iso-butanol is not necessarily related to PLD activity. In this study, we concluded that suppressed activity of the RD29A promoter by iso-butanol is not due to suppression of PLD activity in Arabidopsis plants. Interestingly for us, a previous study found that iso-butanol affects microtubules in Arabidopsis plants (Gardiner et al., 2003). Further analysis is required to clarify the effect of iso-butanol.
Besides the results in the experiment with iso-butanol, we confirmed PLDα1 and PLDδ are major enzymes that form PA in Arabidopsis seedlings as previously described (Uraji et al., 2012) and shown in (Fig. 2.5). However, unlike in stomatal closure, knockout mutants *plda1*, *pldδ*, and *pldα1/δ* do not show any obvious effect in *RD29A* gene expression upon ABA exposure (Fig. 2.10 & 2.11). Kinetics analysis of the promoter activity in the *RD29A::LUC* transgenic plant also indicates that the *RD29A* promoter activity is not alternated with PA exposure (Fig. 2.16).

These results address our first question; why do both knockout and overexpression of a PLD gene upregulate the *RD29A* gene expression? Our results suggest that the higher levels of *RD29A* expression in the *plda1* knockout lines, previously observed in this study (Fig. 2.10a), is not due to alteration of the core components (inhibition of PP2C by PA) in the ABA signaling pathway but other factors such as alteration of membrane composition. Similarly, higher levels of *RD29A* expression by over-expression of PLDα1, which is previously observed (Peng et al., 2010) is most likely caused by alteration of membrane composition but not alteration of the core components in the ABA signaling pathway. A relationship between changes in membrane lipid composition and changes in gene expression was previously identified in Arabidopsis plants (Szymanski et al., 2014), supporting the idea that upregulation of the *RD29A* gene expression is due to changes in the membrane composition but not due to alteration of the ABA signaling pathway.

We also found that exogenously added PA is internalized into inner membranes within minutes in root cells (Fig. 2.17). Exogenously added phospholipids are first incorporated onto the outer leaflet of the plasma membrane and transferred to the inner leaflet of the membrane by enzyme flippase (Davis et al., 2020). Despite the requirement of enzymatic reaction, exogenously added PA is detected in cytosolic area of the cells within minutes. This suggests that PA formed
in the plasma membrane can be spontaneously distributed into the internal membranes in the order of minutes. The double knockout mutant \textit{pld}α1/δ does not show distinguishable distribution of PC and PA, compared to wild type plants (Fig. 2.18).

These results address our second question; Does ABI1 inhibition by PA occur only in the plasma membrane or also in the internal membrane? Our results suggest that the distribution of PC and PA from the plasma membrane into the cytosolic area in the cells would occur independently of ABA exposure and the status of PLDα1 and PLDδ in the plants. Our finding supports the idea that PA inhibition on ABI1 does not require tethering in the plasma membrane (periphery of the cells) but can inhibit ABI1 within the cytosolic area of the cell. Vesicles and internal membranes are difficult to observe by the immunohistochemical method, which was used in the study that concluded ABI1 is tethered in the plasma membrane (Zhang et al., 2004). On the other hand, fluorescent protein tagged ABI1 is more easily detected within the cells by microscopy, which was used in the study that concluded ABI1 is freely mobilized within cells. Either way, both studies support the idea that PA inhibits ABI1 by direct binding.

This also can explain how PA formed by PLDα1 binds and regulates SPHK1/2 that is localized in vacuolar membrane (Pandit et al., 2018). Our analysis suggested that PA formed by PLDα1 in the plasma membrane can be rapidly transferred to the vacuolar membrane. Similarly, findings from a previous study found that PLDα1 was also distributed within cytosol in addition to the plasma membrane (Li et al., 2009) and this agrees with the diverse distribution of NBD-PA (Fig. 2.17). Either way, our study supports an idea that PA can be formed by PLDα1 not exclusively in the plasma membrane but also in the cytosolic area.

Then why does PA affect stomatal closure but not the \textit{ABRE} promoter activity even though the core components are shared? We now believe this is caused by a combination of two
factors. One is the presence of homologous proteins of PP2C that redundantly transduce the ABA signaling to the downstream protein, SnRK2. Genetic studies found 4 homologous proteins, ABI1, ABI2, HAB1, PP2CA redundantly function as a suppressor of the OST1/SnRK2.6 protein that transduces a signal to close stomata and to up-regulate the ABRE promoter (Xue et al., 2008), (Saez et al., 2006), (Nishimura et al., 2007). Among the homologous proteins, only ABI1 is experimentally confirmed as a binding partner of PA (Zhang et al., 2004), (Mishra et al., 2006). Although ABI2 is in the same clade of ABI1 in a phylogenetic analysis, HAB1 and PP2CA are in different clades respectively (Xue et al., 2008). If we assume HAB1 and PP2CA do not bind to PA, it is reasonable that the effect of abolishing the production of PA in the ABA signaling pathway is minimal. Even though the function of ABI1 and ABI2 is inhibited by PA, HAB1 and PP2CA can still function as an ABA signal transducer (Fig. 2.19).

Second is the dual role of PA in stomatal closure. It is found that PA produced by PLDα1 binds not only ABI1 but also RbohD/F, NADPH oxidase (Zhang et al., 2009). The binding of PA to RbohD/F up-regulates the formation of ROS, which is required for stomatal closure but not necessary for the ABRE promoter activation (Zhang et al., 2009), (Song et al., 2014). When PA production by PLD is halted, the production of ROS is also halted (Zhang et al., 2009). This makes stomatal closure impaired. On the other hand, even when PA production is halted, the signal for the RD29A activation is still transduced (Fig. 2.10 & 2.13).

In summary, this study solved a previously unsolved question about the involvement of PA in the ABRE promoter activation. Our study found out that PA is not involved in the activation of the promoter. Impairing stomatal closure by inhibiting or genetically removing PLDα1 is not due to inhibition of the core ABA signaling pathway but inhibition of NADPH oxidase inhibition. It is still possible that PA alters other gene promoters independent from the ABA core signaling
pathway. PA formed by PLD in the plasma membrane would be spontaneously transported to the cytosolic area of the cells within minutes. These results should help to clarify the key role of PA in ABA signaling transduction.

Figure 2.19. A cartoon showing the ABA signaling pathway components and the redundancy in the species involved. Downstream responses of stomatal closure or ABA-induced gene expression could follow any of the homologous species creating redundancy. ABI1 has been shown to be regulated by PA but other PP2Cs could still be active even in the presence of ABA and this would lead to downstream gene induction.
CHAPTER 3.
DEVELOPING A PA BIOSENSOR: SPATIOTEMPORAL ANALYSIS OF PHOSPHATIDIC ACID FORMATION DURING OSMOTIC STRESS IN *ARABIDOPSIS THALIANA*

3.1 Introduction

Phosphatidic acid (PA) is a membrane phospholipid whose function is both signaling and structural. As a signaling molecule PA is involved in responses towards drought, salinity, stress hormone ABA, pathogen attack, wounding, cold, and freezing (Li et al., 2009), (Testerink and Munnik, 2005). Structurally, PA is the simplest phospholipid and is an important precursor for the formation of glycerolipids and phospholipids (Athenstaedt and Daum, 1999), (Stillwell, 2016). Signaling PA is synthesized by a repertoire of enzymes belonging to the phospholipase D (PLD) or phospholipase C/diacylglycerol kinase (PLC/DGK) pathways. Plants have multiple PLD, PLC, and DGK enzymes. For example, in the model plant *Arabidopsis thaliana*, there are 12 PLDs, 9 phosphatidylinositol phospholipases C (*PI-PLC*), 6 non-specific phospholipases C (*NPC*), and 7 DGK genes (Wang et al., 2006). Even with this many enzymes, PA constitutes about 1% of total phospholipids in cells, and its concentration increases within minutes on the onset of stress which is then rapidly hydrolyzed to diacylglycerol (DAG) to halt the signal transduction (Tei and Baskin, 2020).

PA is composed of diverse types of acyl chains in the *sn*-1 and *sn*-2 positions. Different acyl chains are linked to different enzymes and different stress signals. For example, in Arabidopsis, PA species having 34:1, 34:2, 34:3, 36:3, or 36:6 are produced in response to ABA (Hong et al., 2010). The enzymes too have differences in expression, activation, subcellular localization, and substrate specificity. PLDα1 has both cytosolic and membrane-bound localizations while PLDζ2 is found on the tonoplast. PLDδ, PLDe, and PLDα3 are found on the plasma membrane (Li et al., 2009). NPC 1 and 3 are localized to the ER and Golgi apparatus while
NPC4 on the plasma membrane. NPC5 is localized in the cytoplasm and ER in Arabidopsis (Nakamura and Ngo, 2020). PLDα1 was found to be more active in tissues like flowers, siliques, and roots in Arabidopsis (Fan et al., 1999). AtPLC2 protein on the other hand is highly expressed in trichomes of young rosettes, young floral buds, and vascular tissues of leaves, petals, and roots. Subcellular localization study showed that AtPLC2 is found in the plasma membranes in different cell types (Kanehara et al., 2015).

This shows that there would be an elaborate spatiotemporal regulation of PA in cells in response to different stressors which activate different enzymes in different tissues and cellular localizations. However, current knowledge with respect to the spatiotemporal formation of PA in signal transduction is limited (Li et al., 2019). We previously showed that exogenously added PA is distributed both on the plasma and internal membranes, but we did not investigate endogenously produced PA (Chapter 2). Therefore, the creation of a throughput assay to detect PA production in vivo with a short time interval (i.e., minutes) and high sensitivity to detect the small amount of change in PA is necessary to advance the knowledge of PA signaling. The new assay should allow better insight into the spatiotemporal regulation of PA to determine or decipher the different signaling pathways, therefore identifying novel signaling pathways. There have been many assays to understand the role of distinct species of PA in stress response. This includes the use of pld, pi-plc, or plc gene knockout mutant plants, quantifying the increase in PA concentration on the onset of stress using methods such as chromatography (Bargmann et al., 2009), (Holland et al., 2003) radionuclide labeling (Munnik et al., 2000), or mass spectrometry (Bathena et al., 2011). These methods are quantitative but do not provide spatial dynamics of PA formation. This coupled with the fact that PA is usually in low concentrations in cells makes its analysis difficult.
Fluorescent biosensors are excellent tools for high spatiotemporal resolution of cellular activity in real-time (DiPilato and Zhang, 2010), (Balcerowicz et al., 2021). They provide a quantitative analysis of PA formation in relation to a specific stressor. Such assays are also highly sensitive and would detect slight changes in PA produced during stress. They would also determine tissue-to-tissue differences in PA synthesis. Previously, fluorescent biosensors have been used to detect PA synthesis dynamics in plant cells. In a previously published work (Potocký et al., 2014), a PA biosensor was created to study the dynamics of PA formation in growing pollen tube cells. They were able to show an increase in PA formation during the growth of the pollen tube. However, their study was limited to only isolated pollen tubes in the plant. The PA binding domain used to detect PA in the PA biosensor is Soluble N-ethylmaleimide sensitive factor attachment protein receptor (SNARE) protein Spo20p from Saccharomyces. The sensor suffers from high background signals due to diffusion of the biosensor that does not bind to PA in the cytosol. Hence, analysis was limited only to the plasma membrane of isolated pollen tubes. Another research group created a PA biosensor using the Förster resonance energy transfer (FRET) method. The PA binding domain used to detect PA in the PA biosensor was cloned from the Arabidopsis NADPH oxidase respiratory burst oxidase homolog D (RbohD) PA binding residues (Li et al., 2019). This sensor was highly sensitive, and the mutated version of the binding domain did not show any changes in the signal, confirming the efficiency of the sensor and its high specificity. However, the sensor was anchored to the plasma membrane using the C-terminal of K-Ras4B from the guanine nucleotide exchange factor Ras family. Therefore, the sensor only detected changes at the plasma membrane.

Here we present a new fluorescent-based PA biosensor that is intended to detect PA formation in both plasma and internal membranes with low background signals. This biosensor is based on dimerization-dependent fluorescent proteins (Alford et al., 2012), which were previously
used to determine caspase activity and Ca\(^{2+}\) concentration dynamics in Hela cells (Ding et al., 2015). The assay involves the expression of two dark monomers to create a fluorescent dimer.

![Diagram of dimerization-dependent fluorescent proteins](image)

**Figure 3.1.** Principle of dimerization-dependent fluorescent proteins. Monomers GA and RA can interact with monomer B interchangeably with weak interaction ($K_d \approx 40 \mu M$). A dimer of GA-B and a dimer of RA-B then emits green and red fluorescence, respectively.

![Diagram of PA biosensor](image)

**Figure 3.2.** Newly designed PA biosensor. T14, the PA binding domain is fused to RA and B. With low concentration of PA in an area of membrane (on the left of the figure), low intensity of red fluorescence in the membrane and high intensity of green fluorescence are observed. On the other hand, with high concentration of PA in an area of membrane, high intensity of red fluorescence and low intensity of green fluorescence are observed (on the right).

The dark monomers are RA, GA, and B3. RA carries the red fluorophore while GA carries the green fluorophore. These fluorophores are not correctly configured when the protein (RA or
GA) is expressed alone and therefore they do not fluoresce in the monomer form. The B3 monomer is an activator that binds either RA or GA with equal weak affinity (Ding et al., 2015). Upon binding of B3 to RA or GA, the fluorophore in RA and GA is correctly configured and emits red or green fluorescence, respectively (Fig. 3.1). In our PA biosensor, both RA and B3 monomers are fused to T14, the PA binding domain of a protein Trigalactosyldiacylglycerol 4 (TGD4) from Arabidopsis (Wang et al., 2013) (coined RAT14 and B3T14, respectively). TGD4 protein is located on the chloroplast outer envelope and is involved in the transfer of PA from the ER to the chloroplast (Wang et al., 2012). T14 binds PA selectively and strongly (Wang et al., 2013). Since the B3 monomer can bind to either the RA or GA monomers competitively, more red fluorescence would be formed when PA concentrations increase which would lead to a reduced concentration of green fluorescence (Fig. 3.2). By determining the ratios of red to green fluorescence, it would be possible to detect when and where PA is formed within the cell (i.e., both plasma and internal membranes).

3.2 Materials and methods

3.2.1 Molecular cloning and transformation of E. coli

Custom double-stranded DNA coding for RAT14, GA, and B3T14 were purchased from Thermo Fisher Science (sequence information is in the appendix). The DNA fragments were cloned into pBI221 (2003a) and pBI121 (2003b) vector plasmids that possess constitutive Cauliflower Mosaic Virus 35S (CAMV35S) promoter for expression in plants. Restriction enzyme sites BamHI and SacI were used to insert a cDNA encoding RAT14 or B3T14 while BamHI and XhoI were used to insert a cDNA encoding GA downstream of the promoter. For amplification of the plasmids, the cloned plasmids were transformed into E. coli DH5α. Competent DH5α E. coli (Green et al., 2012) were thawed. 50 µL of competent cells were placed in a microcentrifuge tube
with 100 ng/µL of respective plasmid DNA. The tubes with the mix were placed on ice for 20 minutes. The tubes were then placed on a heat block set at 42°C for 1 1/2 minutes. The tube was placed on the ice again and 200 µL SOC media (Green et al., 2012) were added to each tube. The mixture was then placed in a heat block set at 37°C for 30 minutes. Bacteria were plated on LB agar (Green et al., 2012) containing kanamycin antibiotics to select only transformed bacteria. Colonies were then collected and inoculated to obtain a large sample of transformed bacteria. PBI221 and pBI121 plasmids containing RAT14, B3T14, and GA were then extracted from the bacterial cultures using a plasmid extraction kit (Qiagen cat#27104).

3.2.2 Transient protoplast transformation

*Arabidopsis thaliana* leaf mesophyll protoplasts were prepared based on the previously published protocol by Kato lab (Kato and Jones, 2010). The protoplasts were then transformed with 2 µg/µL each of pBI221 plasmids containing RAT14 and B3T14. Alternatively, the protoplasts were transformed with 2 µg/µL each of pBI221 plasmids containing GA and B3T14. The protoplast transformation was conducted using the polyethylene glycol mediated transformation protocol that was previously published (Kato and Jones, 2010). The protoplasts were incubated in the dark at room temperature for 16 hours before microscopy analysis.

3.2.3 Agrobacterium transformation

The pBI121 vector plasmids containing either RAT14, B3T14, and GA were introduced in *Agrobacterium tumefaciens* strain GV3101/pMP90 separately (Koncz and Schell, 1986), (Van Larebeke et al., 1974). 1 µg/µL of the plasmids were used to transform competent Agrobacterium using the freeze and thaw method. 1 µg/µL of plasmid was added to thawed competent Agrobacterium cells and placed on ice for 5 minutes. After 5 minutes the same was placed in liquid nitrogen for another 5 minutes. The plasmid- Agrobacterium mix was then warmed to 37°C for 10
minutes. 1 mL of YEP broth (Green et al., 2012) without antibiotics was then added and the sample incubated at 28°C for 30 minutes with minimal shaking. The mix was then centrifuged at 8,000 rpm for 3 minutes to separate the Agrobacterium cells, the supernatant was removed to leave 200 µL of YEP with Agrobacterium and this was resuspended and plated on YEP agar plates containing 50 µg/mL kanamycin, 10 µg/mL rifampicin, and 25 µg/mL gentamycin antibiotics. The Agrobacterium was then cultured in the dark at 28°C. One colony was picked after two days and cultured in 5 mL LB broth containing the same antibiotics at 28°C to make a glycerol stock.

3.2.4 Transient leaf infiltration assay

Agrobacterium strains containing a plasmid expressing either RAT14, B3T14 or GA were cultured separately as described in (Mangano et al., 2014). A 5 mL culture of Agrobacterium carrying the RAT14 gene was re-suspended in infiltration media. It was then mixed with 5 mL of infiltration media containing Agrobacterium with the B3T14 gene. Another mix was made from 5 mL of infiltration media with Agrobacterium carrying the GA gene mixed with infiltration media containing 5 mL of Agrobacterium with the B3T14 gene. 1 mL of each mix was then loaded into a syringe and used to load the inoculum onto 4-week-old Arabidopsis thaliana (Col) leaves on the abaxial side. Two inoculation sites were made on one leaf on either side of the main vein. A sharpie was used to mark the areas where the inoculations had been made (Mangano et al., 2014). The infiltrated plants were left to grow for two more days in a growth chamber before the leaves were collected for microscopy analysis.

3.2.5 Wide field fluorescence microscopy

For protoplasts imaging, 100 µL of transformed protoplasts were placed on a glass slide (Corning cat#2948) and covered with a coverslip (Corning cat# 2980). Images were captured on a Leica DM6B fluorescence microscope equipped with a 40X/0.85 objective lens. TRITC filter was
used to capture red fluorescence while the FITC filter was used to capture green fluorescence. TRITC filter allows excitation of 532-558 nm and an emission of 570-640 nm. FITC filter allows excitation of 480-500 nm and an emission of 507-543 nm. Transient infiltrated Arabidopsis leaves were placed on a microscope slide (Corning cat#2948) and 100 µL distilled water was poured on top. A coverslip (Corning cat# 2980) was then placed, and the Leica DM6B fluorescence microscope was used for imaging using a 40X/0.85 objective lens and TRITC and FITC filter set to capture both red and green fluorescence.

3.2.6 Transformation of Arabidopsis plants to make transgenic plants

The transfected *Agrobacterium tumefaciens* were used to transform *Arabidopsis thaliana* plants using the floral dip method (Zhang et al., 2006). *Agrobacterium* with RAT14, B3T14, or GA expression cassettes were cultured in YEP separately as described in (Zhang et al., 2006) and the bacteria were resuspended in 5% (wt/vol) sucrose solution with 0.02 % (vol/vol) Silwet L-77. A suspension of 250 mL from each of the different Agrobacterium expressing the three different cassettes was mixed to make a total of 750 mL. This was used to dip inflorescences of 6-week-old Arabidopsis plants. Seeds were harvested from the transformed plants on plant maturation and drying. For growth, seeds were sterilized in 70% Ethanol for 1 minute then in 50% bleach, 0.05% triton for 10 minutes. The seeds were then rinsed 6 times with sterile distilled water, and then plated on 0.7% agar half MS containing 100 µg/mL kanamycin and 100 µg/mL carbenicillin (Zhang et al., 2006). Positive transformants were identified by detecting both green and red fluorescence in roots of the seedlings and these were selected as T1 generation plants. They were then self-crossed, and their seeds were selected again using Kanamycin for the T2 generation. Selected T2 plants with both red and green fluorescence were further self-crossed to obtain T3 generation plants. Seedlings from T3 seeds were used in subsequent assays.
3.2.7 Time course ABA and salt assay with transgenic Arabidopsis seedling and confocal microscopy

Seven-day old seedlings in which red and green fluorescence were detected were placed in a ½ MS solution (Murashige and Skoog, 1962) containing 115 mM NaCl or 200 µM ABA and imaged under a Leica SP8 Confocal microscope equipped with argon and white laser system and a 63x/1.20 water immersion lens. This was used to take optically sliced-and-stacked images every 10-minute for 1 hour. Six seedlings were placed on a glass slide (Corning cat#2948) with ½ MS containing ABA or NaCl. The seedlings were then covered with a coverslip (Corning cat# 2980). Red fluorescence was detected with 572 nm excitation and 630/30 nm emission with a gate time of 0.3 – 12 nanoseconds. Green fluorescence was detected with 480 nm excitation and 535/25 nm emission without gate time. Image scanning conditions were 2048 X 2048 pixels, 12-bit depth, and 400 Hz scan speed. Images were taken in a z-stack of 10 µm with a z-step size of 1µm. Images collected were analyzed using ImageJ (Fiji) software. The fluorescent intensity was determined either on the plasma membrane or the internal membranes using a line scan tool. A line scan was placed on the image where both red and green fluorescence was expressed. Fluorescent intensity was then measured separately for red and green channels. The same line was placed on part of the image without any fluorescence and intensity determined as background intensity. Final fluorescent intensity was calculated by subtracting the background intensity from the red and green intensities. Ratios of red to green were then determined and plotted.

3.2.8 Total lipid extraction and TLC analysis of phospholipids

Leaves (0.2g fresh weight) from four-week-old Arabidopsis plants were floated on ½ MS solution containing 200 µM ABA. As a control, the same weight of leaves was floated on ½ MS with DMSO for 0, 10, 25, 45, and 60 minutes. After the treatment, the leaves were immersed in 3 mL isopropanol with 0.01% butylated hydroxytoluene (BHT) heated at 75°C. The leaves were
incubated in the hot isopropanol for 15 minutes. The hot isopropanol with the leaves was allowed to cool and then 1.5 mL chloroform and 0.6 mL water were added, and the mixture was placed on a digital vortex to mix at 500 rpm using a digital vortex mixer for 1 hour. The extract was collected using a pipette and set aside. Four mL chloroform and methanol mixture (2:1) was added to the leaves for further extraction and the mix was placed on the digital vortex for shaking at 500 rpm for 30 minutes. The mixture was then collected and mixed with the previous extract. Further extraction using the chloroform: methanol mixture was repeated until the leaves turned white. One mL 1M KCl was added to the total extract and the mixture was centrifuged at 3,000 rpm for 10 minutes. The upper phase was discarded, and 2 mL distilled water was added to the lower phase and mixed. The mixture was centrifuged at 3,000 rpm for 10 minutes. The upper phase was again discarded. The lower phase was then evaporated on a heat block at 70°C overnight in the fume hood. The dry extract was then dissolved in 40 µL chloroform for separation on a TLC silica gel glass plate F254 (Millipore Sigma cat# 99571). A solvent of chloroform, methanol, and aqueous ammonia (65:38:8) was used to develop the TLC plate. A PA standard (Sigma cat# P9511) was used on the TLC plate for the quantification of phosphatidic acid. The phospholipid separation on the TLC plate was visualized under UV and an image was taken. ImageJ (Fiji) was used to deduce the amount of phosphatidic acid using relative fluorescent intensities of the samples.

A PA standard curve developed from the PA standard (Sigma cat# P9511) on the TLC with known concentrations was used to quantify the amount of PA in the samples.

3.3 Results.

3.3.1 Expression cassettes of the PA biosensor

Three expression cassettes were created for the PA biosensor. A gene encoding recombinant protein RAT14, B3T14, and GA was expressed by CAMV35S constitutive promoter.
The genes can be expressed transiently in Arabidopsis protoplasts with the pBI212 vector or Arabidopsis leaves and in transgenic Arabidopsis plants with the pBI121 vector.

![Gene constructs for the PA biosensor](image)

**Figure 3.3.** Gene constructs used for the PA biosensor. Gene constructs for the PA biosensor consist of constitutive promoter CAMV35S, NOS terminator, and fluorescent protein monomers RA and GA which only fluoresce in a dimer with B3. RA and B3 monomers are fused to PA binding domain T14 from Trigalactosyldiacylglycerol 4 (TGD4) (Wang et al., 2012).

### 3.3.2 Red and green fluorescence were detected in protoplasts and leaf epidermal cells

Recombinant proteins of RAT14, GA, and B3T14 were expressed simultaneously and transiently in Arabidopsis leaf mesophyll protoplasts or leaf epidermal cells to preliminary confirm expression of red and green fluorescence in plant cells. When vector plasmids containing RAT14 and B3T14 were transfected into the protoplasts, red fluorescence was observed (Fig. 3.4a). On the other hand, when vector plasmids containing GA and B3T14 were transfected into the protoplasts, green fluorescence was observed (Fig. 3.4b). The same was observed when Agrobacterium containing a vector plasmid containing RAT14, B3T14 or GA were co-inoculated in Arabidopsis leaves. When Agrobacterium containing RAT14 or B3T14 were co-inoculated, red fluorescence was observed (Fig. 3.4c). On the other hand, when Agrobacterium containing GA or
B3T14 were co-inoculated, green fluorescence was observed (Fig. 3.4d). This shows that the vectors constructed express the recombinant proteins in Arabidopsis plants and monomer proteins were able to dimerize to produce fluorescence in cells.

![Image of fluorescence in Arabidopsis plants](image)

Figure 3.4. Red and green fluorescence are detected in protoplasts and leaf epidermal cells. In (a) equal amounts of a plasmid containing RAT14 expression cassette and a plasmid containing B3T14 expression cassette were used to transform Arabidopsis protoplasts. In (b) equal amounts of a plasmid containing GA expression cassette and a plasmid containing B3T14 expression cassette were used to transform Arabidopsis protoplasts. For the leaf transient expression, a mixture of Agrobacterium that carries a plasmid containing one of the expression cassettes was used in each case. For the red fluorescence equal amounts of Agrobacterium carrying RAT14 and Agrobacterium carrying B3T14 were used to inoculate the leaves (c). For the green fluorescence equal amounts of Agrobacterium carrying GA and Agrobacterium carrying B3T14 were used to inoculate the leaves (d).
3.3.3 Red and green fluorescence are detected in transgenic plants

After confirming green and red fluorescence, transgenic Arabidopsis plants expressing all the three monomers (RAT14, B3T14, and GA) were created by the floral dip method (Zhang et al., 2006). Expression cassettes of RAT14, B3T14, and GA were simultaneously introduced into the plant by mixing equal amounts of the three independent Agrobacteria that carry each of the expression cassettes. This allows simultaneous transformation of a plant with independent exogenous genes (Padilla-Guerrero and Bidochka, 2017). Two T1 plants, in which both red and green fluorescence were detected, were self crossed to obtain 15 independent T2 plants in which both red and green fluorescence were detected. Three T2 plants were self-crossed to obtain 27 independent T3 plants in which both red and green fluorescence were detected. T3 generation plants were used for further analysis. While red and green fluorescence were detected in the transgenic plant leaves (Fig. 3.5b & c) no red and green fluorescence was detected wild type plants (Fig. 3.5j & k). Red and green fluorescence was also detected in the root tissue of the transgenic plants (Fig. 3.5f & g). Green fluorescence was observed in the cytosol of both the leaf and root cells (Fig. 3.5c & g). On the other hand, red fluorescence was observed in both the cytosol and in the plastids and colocalized with chlorophyll fluorescence (Fig. 3.5b, d, f, & h) in the leaves and roots. This suggested the T14 binding domain that was fused to the RA and B3 monomers may have a weak chloroplast localization signal. These plants were used in subsequent studies on the effect of salt and abscisic acid hormone.
Figure 3.5. Transgenic Arabidopsis express both red and green fluorescence. Red and green fluorescence is detected in transgenic plant leaves (b,c) and roots (f,g) transformed with expression cassettes of RAT14, B3T14, and GA. Chlorophyll autofluorescence was also determined (d,h,l). WT plants show no red or green fluorescence (j,k). Transmission light images were also taken (a, e, i).
3.3.4 Fluorescent ratios increase in the plasma membrane of Arabidopsis roots when exposed to ABA or NaCl (salt), but fluorescent ratios increase in internal membranes only with ABA but not with NaCl (salt).

The transgenic plants were analyzed for the spatiotemporal formation of PA during salt (NaCl) and ABA exposure. Areas of the plasma membrane were defined as a rim of a cell that was recognized with green fluorescence. On the other hand, areas of the inner membrane were defined as a string-like structure that was recognized with red fluorescence within the cytosolic area of a cell. A line scan tool as shown in (Fig 3.6a and 3.6b) was used to determine fluorescent intensity on plasma or internal membranes.

![Figure 3.6. Red and green fluorescence intensities determination. A line scan tool was used to determine the fluorescent intensities. The line tool was placed as shown in (a) and (b) on both red and green images as indicated by the yellow line for fluorescent detection on the plasma membrane.](image)

When 115 mM salt (NaCl) (Fig. 3.7a & b) was exposed to plants, the red to green ratio increased and peaked at around 30 minutes in the plasma membrane. For control, there was a progressive increase in the fluorescent ratios with the highest at 60 minutes after exposure to water (Fig. 3.7a). However, in the internal membranes, fluorescence ratios fluctuated during the observation (Fig. 3.7b).
When the plants were treated with 200 µM ABA (Fig. 3.8a & b) the fluorescence ratios also increased and peaked at around 30 minutes in the plasma membrane. On the other hand, the fluorescence ratios gradually increased during the 60 minutes’ observation with water (Fig. 3.8a). In the internal membrane, an increase in the ratio was observed and peaked at 60 minutes while with water exposure, the fluorescence ratios fluctuated (Fig. 3.8b).

Figure 3.7. Red to green, fluorescence ratios increased on the plasma membrane but not on the internal membranes with 115 mM NaCl (salt). Seedlings were exposed to 115 mM NaCl, or water and images were taken at 10-minute intervals for 65 minutes. Ratios of the fluorescence intensities (red to green) were then analyzed at the area of the plasma membranes (a) and the area of the internal membrane (b). A total of 7 cells in 3 plants were analyzed for NaCl (n=7) and 5 cells in 3 seedlings for control (n=5). The graphs show an average of the fluorescence intensity ratios with error bars from standard error from the mean.
Figure 3.8. Red to green, fluorescence ratios increased on both the plasma membrane and internal membrane with 200 µM ABA. Seedlings were exposed to 200 µM ABA or water and images were taken at 10-minute intervals for 65 min. The ratio of the fluorescence intensities (red to green) was then analyzed at the area of the plasma membranes (a) and the area of the internal membrane (b). A total of 10 cells in 3 plants were analyzed for ABA (n=10) and 5 cells in 3 seedlings for control (n=5) on the plasma membrane. A total of 3 cells from 2 plants were analyzed for ABA (n=3) and 4 cells from 2 plants for control on the internal membranes (n=4). The graphs show an average of the fluorescence intensity ratios with error bars from standard error from the mean.

### 3.3.5 Total amount of PA increases on exposure to ABA and peaks at 30 minutes

To determine changes in the total amount of PA in plants, total lipids were extracted from leaves of 4-week-old Arabidopsis and analyzed on a TLC plate (Fig. 3.9). A standard curve of PA derived from fluorescent intensities of a PA standard on the TLC plate was used to determine PA concentration in the samples (Fig. 3.9). PA amount increased on exposure to 200µM ABA and peaked between 30 and 40 minutes (Fig. 3.10). This increase somewhat agreed with that in the
plasma membrane of a root cell determined with the PA biosensor during ABA exposure.

Figure 3.9. Lipids were extracted and analyzed by TLC. A TLC plate is shown on the left with separated phospholipids extracted from plants incubated with and without 200 µM ABA at different time points and a PA standard of different concentrations. A PA standard curve created from the PA standard is shown on the right.

Figure 3.10. PA concentration from 0.2 g of Arabidopsis leaves increases on incubation with 200µM ABA when compared to the control (water). Total lipids were extracted from leaves treated with and without ABA and the concentration of phosphatidic acid was determined by TLC and the concentration per (g) of fresh weight was determined using the PA standard curve. The concentration of PA increases and peaks between 30 and 40 minutes after incubation.
3.4 Discussion.

Here we presented the use of a new PA biosensor in Arabidopsis plants, which can be used to observe spatiotemporal changes of PA formation. This PA biosensor allows the detection of changes not only in the plasma membrane but also in the internal membranes which were not possible in the previously developed PA biosensors (Potocký et al., 2014), (Li et al., 2019). We did not observe red fluorescence signals in transgenic Arabidopsis that were transformed with only RAT14 nor with only GA (data not shown), confirming dimerization-based fluorescence. However, further control experiments are required to confirm the proper function of this newly developed PA biosensor. Since the sensor is dependent on the ratio between the red and green fluorescence, it is important to confirm that there are equal amounts of protein being expressed. A western blot assay could be used to confirm that equal amounts of the proteins are expressed in the transgenic plants for further analysis. The T14 protein that was used to detect PA in this work appears to contain a weak chloroplast targeting signal because green fluorescence was not detected in chloroplasts. The T14 protein is the PA binding domain of TGD4 that transports PA from the ER to chloroplasts which was localized in the outer envelope of chloroplasts (Wang et al., 2012). Perhaps, on the PA binding site of PA (T14 fragment) remains a part of chloroplast transport signal sequence (Wang et al., 2013). Use of a different binding domain of PA e.g., RbohD (Li et al., 2019) may prevent the accumulation of RAT14 and B3T14 in chloroplasts in further analysis. In addition, in this study, we were not able to clarify the type of internal membranes (i.e., Golgi, ER, or vacuole) due to the lack of a fluorescent label that uniquely marks each organelle in the transgenic plants. Further development, such as marking an organelle with a cyan fluorescent protein in the transgenic plant that expresses the PA biosensor, is required to identify the internal membranes that emit red fluorescence.
Nevertheless, we showed, for the first time, an increase of PA formation in internal membranes in addition to the plasma membrane by ABA exposure (Fig. 3.8a & b). This agrees with another finding described in Chapter 2. Interestingly for us, an increase of PA formation in internal membranes was not observed by NaCl exposure although NaCl exposure increases PA formation in the plasma membrane (Fig. 3.7a & b). The different responses between exposures of salt and ABA suggest that distinct signaling pathways may exist based on the localization of PA. We also found changes of total PA amount during ABA exposure quantified by the TLC analysis were somewhat similar to the changes of PA quantified by the PA biosensor (Fig. 3.8a & b). Although the TLC analysis was conducted only once, similar changes of a total PA have been reported previously (Zhang et al., 2004). This suggested that an increase of PA formation may occur differentially, depending on the locations or types of the membrane, underlining the importance of the use of the PA biosensor developed in this study.

On confirming the proper functionality of the PA biosensor with further characterization and improvement, this would be a powerful tool to discriminate the location and time point when transient PA is formed on the onset of stress. It would advance knowledge of PA signal transduction and determine any de novo signaling pathways that PA is involved in. In addition, it would also help to reevaluate already known signaling pathways. The biosensor also should be able to determine signaling of other stressors other than NaCl and ABA such as wounding and pathogen defense in the future.
CHAPTER 4.
A DYNAMIC MODEL OF THE ABA SIGNALING PATHWAY PREDICTS
TRANSLATIONAL REGULATION OF PP2C IS IMPORTANT IN THE
RESPONSE KINETICS OF THE PATHWAY.

4.1 Introduction

The Abscisic acid (ABA) signaling pathway is a well-characterized signal transduction pathway, leading to downstream ABA responses such as stomatal closure and ABA-induced gene expression (Ng et al., 2014), (Fujii et al., 2009), (Umezawa et al., 2009). The most upstream of the core components in the ABA signaling pathway are ABA-receptors named pyrabactin resistance/pyr1-like/ regulatory components of ABA receptors (PYR/PYL/RCAR) that bind ABA and in turn interact with protein phosphatases PP2Cs, aba insensitive1/2 (ABI1/ABI2), hypersensitive to aba1 (HAB1/HAB2), and clade A PP2Cs, aba-hypersensitive germination 1/3 (AHG1/3), and highly aba induced 1/2/3 (HA1/2/3). Without the PYR interaction, these PP2Cs inhibit SnRK2 kinases SnRK2.2, SnRK2.3, and SnRK2.6 (Vishwakarma et al., 2017), (Ma et al., 2009), (Park et al., 2009), (Yin et al., 2009), (Melcher et al., 2009), (Nishimura et al., 2009), (Santiago et al., 2009), (Soon et al., 2012). The SnRK2 kinases phosphorylate ABRE binding factors 1/2/3/4 (ABF1/2/3/4) which bind ABA-responsive elements (ABREs) on ABA-induced genes (Reeves et al., 2011), (Furihata et al., 2006), (Johnson et al., 2002). Alternatively, the SnRK2 kinases phosphorylate the slow-anion channels (SLAC1) leading to their activation and subsequently leading to stomatal closure due to anion and K⁺ efflux and eventual solute loss from the guard cells (Albert et al., 2017), (Lee et al., 2009).

The ABA signaling pathway has been modeled using mathematical equations to help understand the ABA signaling pathway in guard cells leading to stomatal closure (Maheshwari et al., 2019), (Maheshwari et al., 2020), (Li et al., 2006), (Albert et al., 2017). These works have led to the determination of new predictions and hypotheses in the ABA signaling pathway, for
example, the role of feedback regulation, ROS, Ca\textsuperscript{2+}, pH, and heterotrimeric G-protein signaling in ABA-induced stomatal closure (Maheshwari et al., 2019), (Li et al., 2006), (Albert et al., 2017). In addition, the additive effect of ABA and salt stress on ABA and drought-responsive expression of genes was also explained using mathematical modeling (Lee et al., 2016).

Recent discoveries in the ABA-signaling pathway include other molecules e.g., phosphatidic acid (PA) that interact with components in the core signaling pathway in addition to feedback regulation. The feedback regulation involves upregulation of PP2Cs gene expression by ABA, which would result in the deactivation of ABFs and SnRK2 kinases (Saez et al., 2004), (Wang et al., 2019),(Fujita et al., 2009). It also includes the upregulation of ABF-genes by ABA, which would result in more expression of ABF (Wang et al., 2019). All these create a diverse feedback loop that regulates the signaling pathway.

In addition, the target of rapamycin (TOR) protein kinase was shown to phosphorylate PYL ABA receptors in absence of stress to inhibit ABA stress response. On the other hand, SnRK2 kinases phosphorylate Raptor, which associates with TOR, leading to TOR kinase inhibition (Wang et al., 2018). In another study, TOR was found to suppress ABA-responses by phosphorylating Arabidopsis thaliana yet another kinase (AtYAK1) (Forzani et al., 2019) which is a positive regulator of ABA-mediated signal responses (Kim et al., 2016). Therefore, TOR was proposed to be a negative feedback regulator for the ABA signaling pathway.

Another feedback mechanism discovered is the E3-ligases that have been shown to interact and, in some cases, lead to the degradation of ABA signaling components like PP2CA (Wu et al., 2016), SnRK2.6 (Ali et al., 2019), and PYL5/7/8/9 (Zhao et al., 2017) thus regulating ABA signaling. In addition, MAPKK-kinases were shown to be important in the activation and phosphorylation of SnRK2 kinases after release from their inhibition by PP2C phosphatases.
(Takahashi et al., 2020) arguing that previously reported autophosphorylation of SnRK2 kinases is insufficient for the relay of ABA signal (Belin et al., 2006).

How the additional components other than PA (previously discussed in chapter 2) together with the feedback mechanisms affect the dynamics of the signaling pathway and downstream activation of the ABF transcription factors leading to enhanced gene expression is little understood. To understand better their role on the ABA signaling pathway we are using dynamic modeling. Dynamic modeling is a powerful tool that enables the assembly of extensive experimental data of individual molecules in a pathway in order to create a better understanding of the signaling pathway dynamics and make novel hypotheses and predictions (Aldridge et al., 2006), (Thakar et al., 2007), (Janes and Yaffe, 2006), (Poolman et al., 2004). In vitro parameters for many of the interactions of the components in the pathway have been experimentally determined previously and this has allowed us to create the ABA signaling pathway in a dynamic model.

The purpose of this study is to build a dynamic model consisting of the core components of the ABA-signaling pathway that leads to the activation of the ABF transcription factors and subsequent gene expression. Identification of reasonable and approximate parameters in the model, but not to perfectly curve fit the model output to the actual plant data, is the focus. We wish to provide a model that allows researchers to look at the dynamics of the core components in the ABA signaling pathway and investigate the connectivity of a molecule of their interest in the pathway. In this report, we describe how we built, optimized, and verified the model. We then report that a new hypothesis based on the model that agrees with preliminary results in actual experiments. Our model suggests that modification of translational rate in the PP2C proteins and their feedback regulation is the next frontier to understand the ABA signaling pathway.
4.2 Materials and Methods

4.2.1 Building, optimization, and verification of dynamic models

A Previous study defined a minimal set of core components that activate the ABF transcription factor leading to gene expression in the ABA signaling pathway (Fujii et al., 2009). The components are ABA, PYR/PYL/RCAR, PP2Cs (ABI1/ABI2 or HAB1/HAB2), SnRK2.2/3/6, and ABF1/2/3/4 (Fig. 4.1). A recent study identified PP2CA dephosphorylates phosphorylated ABF (Wang et al., 2019). In addition, another study identified MAP3K phosphorylates SnRK2s. (Takahashi et al., 2020). Hence, we added these two in the minimal set of components (Fig. 4.1). We also included the feedback regulation in which the expression of PP2C, PP2CA, and ABF genes are upregulated by the ABRE promoter activity (Wang et al., 2019). A set of 20 ordinary differential equations representing biochemical reactions of each molecule were constructed based on the law of mass action. Homologous proteins that redundantly function in the pathway are considered as a single protein. Initial values of variables and values of parameters in the equations were curated through a literature review of previously published data. The equations, initial values, and parameter values were then compiled and numerically analyzed with MATLAB R2020b SimBiology (MathWorks) with its default setting otherwise specified.

In the model, we assumed:

- ABA signal transduction occurs through molecule-molecule interactions and Michaelis-Menten enzymatic reactions with the core components only in a cell.
- \( K_M = \frac{k_{off} + k_{cat}}{k_{on}} \), in which \( K_M \) is Michaelis constant in an enzymatic reaction, \( k_{off} \) is a dissociation rate constant, \( k_{cat} \) is a catalytic rate constant, and \( k_{on} \) is an association rate constant.
- All molecules freely diffuse in a cell.
A volume of a cell is 50 µL.

A molecule associates with another molecule at the same rate constant, $k_{on} = 1000 \, \mu M^{-1} S^{-1}$, in a cell.

A protein is generated by reactions of gene expression and protein translation, then subjected to degradation.

A concentration of a protein in a cell remains at 0.1 µM at a steady state without ABA activation and feedback regulation.

A gene (mRNA) is expressed from a pair of gene loci that have a constitutively active promoter, then subjected to degradation in a cell.

A gene (mRNA) that is expressed by a feedback regulation has an additional regulatory element (ABRE) in the same gene loci that has a constitutively active promoter.

In numerical analysis, the model was first run for 300 equivalent hours with the ABA variable set at 0 µM so that the system reached a quasi-steady state without the effect of ABA. The variable ABA (100 µM) was then added to the system to track changes of other variables from the quasi-steady state for another 300 equivalent hours. The results showing numerical changes of variables were exported to an Excel file (Microsoft). Necessary data were extracted and presented as Excel graphs. In this report, the time point when the ABA variable was added to the system is presented as time zero.

4.2.2 Optimization of parameters, verification of the model, and prediction of gene expression in plants

To optimize and verify the model, we compared changes of variables in the model to those in the experimental data that we obtained by conducting the experiments or data that were previously published. Using Interactive Exploration Tools in SimBiology, parameters were manually changed so that changes of variables in the model were qualitatively similar to the
experimental data. To verify the model, we alternated parameters and quantitatively evaluated changes in the variable abre.gene, which represents accumulated mRNA expressed from the ABRE promoter, in the model. Fold changes identified in the analysis were compared to data previously published.

**4.2.3 Sensitivity analysis**

To analyze what parameter affects changes of the variable abre.genes in a cell, we conducted sensitivity analysis using a function of Calculate Sensitivity in Model Analyzer in SimBiology with the optimized parameters shown in (Table 4.2).

**4.2.4 Transgenic plant and growth conditions**

Transgenic *Arabidopsis thaliana* seeds (CS67900) that carry an *RD29A::LUC* gene expression cassette in the genome and transgenic *Arabidopsis thaliana* seeds (CS25237/CS25230) that carry a *CAMV35S::LUC gene* expression cassette in the genome were obtained from Arabidopsis Biological Resource Center (ABRC). For growth, they were sterilized in 70% Ethanol for 1 minute then in 50% bleach, 0.05% triton for 10 minutes. The seeds were then rinsed 6 times with sterilized distilled water and then plated on ½ Murashige-Skoog (MS) medium containing 0.8% agar. They were then stratified at 4°C for 3 days, then placed in a growth chamber under a growth cycle of 16 hours day 8 hours dark with the light set at 100 µmol m⁻² s⁻¹ and at 22°C.

**4.2.5 Luminescence assay with transgenic *Arabidopsis thaliana***

Twenty-five-day-old transgenic *Arabidopsis thaliana* plants (CS67900) (*RD29A::LUC*) on an agar plate were sprayed with 200 µM ABA and for control with the same volume of DMSO for different time periods (0, 5, 8, 12, and 24 hours). Seedlings (15) were then harvested one hour before the start of the dark cycle and frozen immediately in liquid N₂ and kept at −80°C until analysis. For analysis, frozen seedlings
were ground using a mortar and pestle to form a powder. The powder was then mixed with passive lysis buffer (Promega: Cat. #E1941), the mix was then vortexed and centrifuged at 14,000rpm for 10 minutes. 40 µL of the supernatant was placed in a well on a 96 well plate (Thermo Fischer cat# 267350) and mixed with 100 µL luciferase assay substrate (Promega: Cat. #E151A). Relative Luminescence Unit (RLU) was detected using a Veritas™ microplate luminometer. 3 RLU readings were made for each well and then averaged as data for one replicate.

4.2.6 Rapamycin treatment of transgenic *Arabidopsis thaliana*.  

One-week-old transgenic *Arabidopsis thaliana* seedlings (CS67900) expressing *LUC* under the *RD29A* promoter and (CS25237/CS25230) expressing *LUC* under the *CAMV35S* promoter were placed in wells in a 96-well plate and incubated with either 200 µM ABA only or 200 µM ABA and 10 µM rapamycin (Millipore-Sigma cat#53123-88-9) or with 10 µM rapamycin only. With the *RD29A::LUC* transgenic plants, 3 seedlings were placed in each well and for the *CAMV35S::LUC* transgenic plant, 1 seedling was placed in each well. Each replicate had 6 wells for each treatment. RLU was detected using 1mM of D-luciferin (ThermoFisher: Cat. #88293) as the substrate and a Veritas™ microplate luminometer. Three RLU readings were made every hour for 8 hours continuously.
Figure 4.1. A schematic model showing core components in the ABA-signaling pathway and key interactions and parameters. Core components and their interactions are shown using squares and arrows, respectively. The arrows represent molecular interactions or catalytic reactions. Parameter names for the different reactions are shown as $k_f$ or $k_r$ with a unique number for each reaction. The values of each parameter are shown in Table 4.1.

Key:
- ABA = Abscisic acid.
- PYR = ABA receptors.
- PP2C = Protein phosphatase 2C.
- SnRK2 = SnRK2 kinase.
- SnRK2-P = phosphorylated SnRK2 kinase.
- ABF = ABRE-binding transcription factors.
- ABF-P = phosphorylated ABF.
- ABRE = ABA responsive element.
- Molecule-molecule interaction.
- Michaelis Menten catalysis reaction.
- Translation.
- ABA response gene expression.
- Constitutively expressed protein.
- Phosphorylated protein.
- ABA.
- ABRE promoter.
4.3 Results

4.3.1 Initial values of variables and initial guess of parameters were determined through literature curation

We curated previously published data to define an initial value of variables and parameters in the model of the ABA signaling pathway that activates the ABF, resulting in activation of the ABRE promoter. The summary of our curation is shown below (Table 4.1). While defined parameters in protein-protein interactions and enzymatic reactions related to the ABA signaling pathway were mainly found in in vitro studies using recombinant proteins, no studies related to parameters of gene expression, protein translation, and their degradation were found for the ABA signaling pathway. To this end, we referred to parameters from studies using non-plant eukaryotic organisms. These three parameters have a wide range to select from:

1. The equilibrium dissociation constant between ABF-P (phosphorylated ABF) and the ABRE promoter, from 2 nM to 2 µM (Geertz et al., 2012).
2. The transcription rate of the ABRE promoter from 0.01 to 100 hour$^{-1}$ (Hausser et al., 2019).
3. The translation rate of protein from mRNA expressed by the ABRE promoter is from 10 to 10,000 hour$^{-1}$ (Hausser et al., 2019).

The initial values selected for translation and transcription rate constant were 4.5 hr$^{-1}$ and 1 hr$^{-1}$ respectively. This was to ensure that the initial concentration of the proteins was 0.1 µM which is an assumed averaged protein concentration in a cell (Milo and Phillips, 2015).
Table 4.1. Curated values from literature and the values chosen as parameters for the model. Each of the different reactions in the model is shown below with the respective parameters and the source. Parameters in blue were optimized to create transient abre.genes variable accumulation.

<table>
<thead>
<tr>
<th>Description</th>
<th>Reference.</th>
<th>Values found in the reference or assumed.</th>
<th>Optimized parameter values selected in the model.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transcription of all genes.</td>
<td>(Hausser et al., 2019)</td>
<td>Average transcription rate is 1 hour(^{-1}) in Hela cells.</td>
<td>Kf1= 1 hr(^{-1})</td>
</tr>
<tr>
<td>Translation of all genes.</td>
<td></td>
<td>Assuming steady-state concentration of non-feedback regulated protein is 0.1 (\mu)M in a cell.</td>
<td>Kf2= 4.5 hr(^{-1})</td>
</tr>
</tbody>
</table>
| ABA and PYR binding.                   | (Dupeux et al., 2011), (Milo and Phillips, 2015) | \(K_D= 65 \mu\)M                                          | Kf3= 1000 \(\mu\)M\(^{-1}\) s\(^{-1}\)  
|                                        |                                   | \(k_{cat} = 0.924 \) s\(^{-1}\)  
|                                        |                                   | \(K_M = 0.097 \) uM                                              | Kr3= 65000 s\(^{-1}\) \n| PP2C and SnRK2 binding.                | (Milo and Phillips, 2015), (Xie et al., 2012) | \(k_{cat} = 0.924 \) s\(^{-1}\)  
|                                        |                                   | \(K_M = 0.097 \) uM                                              | Kf4= 1000 \(\mu\)M\(^{-1}\) s\(^{-1}\)  
|                                        |                                   |                                                                   | Kr4=0.1 s\(^{-1}\) \n| PP2C and SnRK2-P binding.              | (Milo and Phillips, 2015), (Xie et al., 2012) | \(k_{cat} = 0.924 \) s\(^{-1}\)  
|                                        |                                   | \(K_M = 0.097 \) uM                                              | Kf5= 1000 \(\mu\)M\(^{-1}\) s\(^{-1}\)  
|                                        |                                   |                                                                   | Kr5=97 s\(^{-1}\) \n
“(table cont’d)”
<table>
<thead>
<tr>
<th>Description.</th>
<th>Reference.</th>
<th>Values found in the reference or assumed.</th>
<th>Optimized parameter selected values in the model.</th>
</tr>
</thead>
<tbody>
<tr>
<td>SnRK2 and MAP3K binding.</td>
<td>(Milo and Phillips, 2015), (Ghose, 2019)</td>
<td>$k_{cat} = 14 \pm 2 \text{ s}^{-1}$</td>
<td>$K_f = 1000 \text{ uM}^{-1} \text{s}^{-1}$ $K_r = 23000 \text{ s}^{-1}$</td>
</tr>
<tr>
<td>Phosphorylation of SnRK2 by MAP3K.</td>
<td>(Ghose, 2019)</td>
<td>$k_{cat} = 14 \pm 2 \text{ s}^{-1}$</td>
<td>$K_f = 14 \text{ s}^{-1}$</td>
</tr>
<tr>
<td>Snk2-P and ABF binding.</td>
<td>(Milo and Phillips, 2015), (Xie et al., 2012)</td>
<td>$k_{cat} = 0.04 \text{ s}^{-1}$ $K_M = 19.3 \text{ uM}$</td>
<td>$K_f = 1000 \text{ uM}^{-1} \text{s}^{-1}$ $K_r = 19300 \text{ s}^{-1}$</td>
</tr>
<tr>
<td>PYR.ABA binding PP2C.</td>
<td>(Dupeux et al., 2011), (Milo and Phillips, 2015)</td>
<td>$K_D = 30 \text{ nM}$</td>
<td>$K_f = 1000 \text{ uM}^{-1} \text{s}^{-1}$ $K_r = 30 \text{ s}^{-1}$</td>
</tr>
<tr>
<td>PYR.ABA binding PP2C.SnRK2.</td>
<td>(Dupeux et al., 2011), (Milo and Phillips, 2015)</td>
<td>$K_D = 30 \text{ nM}$</td>
<td>$K_f = 1000 \text{ uM}^{-1} \text{s}^{-1}$ $K_r = 30 \text{ s}^{-1}$</td>
</tr>
<tr>
<td>ABF-P binding PP2CA.</td>
<td>(Milo and Phillips, 2015), (Pan et al., 2015)</td>
<td>$K_M = 11.15 \text{ uM}$ $k_{cat} = 1.04 \text{ s}^{-1}$</td>
<td>$K_f = 1000 \text{ uM}^{-1} \text{s}^{-1}$ $K_r = 11150 \text{ s}^{-1}$</td>
</tr>
<tr>
<td>ABF-P binding ABRE.</td>
<td>(Geertz et al., 2012)</td>
<td>$K_D = 10^{-7} \text{ M}$</td>
<td>$K_f = 1000 \text{ uM}^{-1} \text{s}^{-1}$ $K_r = 1 \text{ s}^{-1}$</td>
</tr>
</tbody>
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<table>
<thead>
<tr>
<th>Description.</th>
<th>Reference.</th>
<th>Values found in the reference or assumed.</th>
<th>Optimized parameter values selected in the model.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Release of SnRK2 from ABA.PYR.PP2C. SnRK2 complex.</td>
<td>(Milo and Phillips, 2015)</td>
<td>$k_{cat} = 10 \text{ s}^{-1}$</td>
<td>kf13=10 s$^{-1}$</td>
</tr>
<tr>
<td>Dephosphorylation of SnRK2-P.</td>
<td>(Xie et al., 2012)</td>
<td>$k_{cat} = 0.924 \text{ s}^{-1}$</td>
<td>kf14=0.9240 s$^{-1}$</td>
</tr>
<tr>
<td>Phosphorylation of ABF by SnRK2-P.</td>
<td>(Xie et al., 2012)</td>
<td>$k_{cat} = 0.04 \text{ s}^{-1}$</td>
<td>$K_M = 19.3 \text{ uM}$</td>
</tr>
<tr>
<td>Dephosphorylation of ABF-P.</td>
<td>(Pan et al., 2015)</td>
<td>$K_M = 11.15 \text{ uM}$</td>
<td>$k_{cat} = 1.04 \text{ s}^{-1}$</td>
</tr>
<tr>
<td>Transcription of ABA induced genes.</td>
<td>(Hausser et al., 2019)</td>
<td>Transcription rate range 0.01 – 100 hr$^{-1}$</td>
<td>kf17=100 hr$^{-1}$</td>
</tr>
<tr>
<td>Translation of feed-backed ABFs.</td>
<td></td>
<td>Assuming steady-state concentration of non-feedback regulated protein is 0.1 μM in a cell.</td>
<td>kf18= 4.5 hr$^{-1}$</td>
</tr>
</tbody>
</table>

“cont’d”
<table>
<thead>
<tr>
<th>Description.</th>
<th>Reference.</th>
<th>Values found in the reference or assumed.</th>
<th>Optimized parameter values selected in the model.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Translation of PP2Cs in the feedback loop.</td>
<td></td>
<td>Assuming steady-state concentration of non-feedback regulated protein is 0.1 μM in a cell.</td>
<td>Kf19 = 4.5 hr⁻¹</td>
</tr>
<tr>
<td>Degradation of mRNAs.</td>
<td>(Hausser et al., 2019)</td>
<td>mRNA degradation in HEK293 cells = 0.06 hr⁻¹</td>
<td>Kf20 &amp; 21 = 0.06 hr⁻¹</td>
</tr>
<tr>
<td>Degradation of proteins.</td>
<td>(Hausser et al., 2019)</td>
<td>Protein decay rate in Hela cells = 0.04 hr⁻¹</td>
<td>Kf22- 38 = 0.05 hr⁻¹</td>
</tr>
</tbody>
</table>

4.3.2 Parameters for transcription rate of the ABRE promoter and binding affinity between ABF-P and the ABRE promoter were optimized so that kinetics of gene expression between the model and actual plants qualitatively resembled.

To understand the connectivity of molecular reactions and effects of parameters in the ABA signaling pathway, we compared the kinetics of gene expression in the model and experimental data in actual plants. Namely, we compared the simulation data to four independent plant data sets that were obtained by four independent research groups. One set was obtained by us using transgenic Arabidopsis thaliana that carries the RD29A::LUC gene cassette used to study the activity of the ABRE promoter. The other three were obtained from previously published data from other research groups that show a change in RD29A gene expressed from a native ABRE promoter.
in the genome of either *Arabidopsis thaliana* (Lee et al., 2016), (Song et al., 2016) or *Oryza sativa* (Singh et al., 2015). Kinetics of the gene expressions up to the first 24 hours in the actual plants and ABA-induced gene expression in the model (variable name abre.genes) were compared (Fig. 4.2).

Experimental data from transgenic *RD29A::LUC* Arabidopsis plants showed transient expression with an initial increase and then a decrease after 5 hours (Fig. 4.2a). Similar transient expression of the *RD29A* gene was also observed in non-transgenic plants, Arabidopsis, and rice (Fig. 4.2b, c, & d) (Lee et al., 2016), (Song et al., 2016), (Singh et al., 2015). When we first simulated the kinetics of abre.genes in the model without the feedback regulation on PP2C, ABF, and PP2CA and no optimization of other parameters, the kinetics were logarithmic upon adding ABA, (Fig. 4.2e). We then simulated the gene expression by adding the feedback regulations in the model (Fig. 4.2f), and further optimized the parameters so that the kinetics of the gene expression in the model qualitatively resembled that in actual plants (Fig. 4.2g). We namely alternated the two parameters, the dissociation rate constant between the activated form of ABF (variable ABF-P) and the *ABRE* promoter (variable ABRE) (parameter kr12) as well as the transcription rate constant of the *ABRE* promoter (parameter kf17) (Fig. 4.1).
Figure 4.2. Transient increase of ABA-induced gene in experimental data in comparison to model output. Kinetics of luciferase activity in the *RD29A::LUC* plant after exposing to 200µM ABA (a). Data is from the mean of three replicates and error bars represent standard error from the mean. Kinetics of *RD29A* gene accumulation in the previously published data with 50 µM ABA (Singh et al., 2015) (b), 100 µM ABA (Lee et al., 2016) (c), and 10 µM ABA (Song et al., 2016) (d). Model output is shown in (e) – (g). In (e) the output shown is from the model without any optimizations or feedback regulation, in (f) feedback regulations were added and in (g) the rates of transcription and ABF-P-ABRE binding parameter were optimized to create the transient output.

4.3.3. Approximation of the model to actual plants was validated

To validate the model, we first compared ABA-dose-dependent response in abre.genes (Fig. 4.3) and luciferase activity in the *RD29A::LUC* transgenic plants (Fig. 4.4). In the model, changes of variable abre.gene are increased in an ABA-dose-dependent manner. In the *RD29A::LUC* transgenic plants, changes in luciferase activity also increased in an ABA-dose-dependent manner. These suggested that the model is approximated to actual plants with respect
to ABA sensitivity although gene expression in the model is more sensitive to a lower concentration of ABA compared to that in the actual plants.

Figure 4.3. The abre.genes variable increases with an increase in concentrations of ABA. Different concentrations of ABA (10, 50, 100, and 200 µM) were used for simulation.

Figure 4.4. ABRE promoter activity increases with increased ABA concentration in actual plants. Luciferase activity in 25-day-old RD29A::LUC plants was determined at 5 hours after spraying different concentrations of ABA, 50, 100, and 200 µM or with DMSO only (0 µM) onto the plants. The bars represent the mean relative luminescence of three replicates with error bars representing standard error from the mean.
We also validated changes of the abre.genes variable in gene-knockout simulations. Namely, we simulated the expression of abre.genes in gene null-mutations of *pyr, pp2c, snrk2*, and *abf*, which were previously studied (Gonzalez-Guzman et al., 2012), (Rubio et al., 2009), (Yoshida et al., 2015). We mimicked the mutations in the model by setting the transcription rate constant 0 for each gene. The mimicked *pyr* null mutant (Fig. 4.5) showed reduced levels of the abre.genes variable and there was no significant difference with and without ABA. Calculated concentrations were 0.00027 µM and 0.00026 µM with and without ABA, respectively. Experimental data shows that *pyr* null-mutants are impaired in ABA-induced gene expression and are insensitive to ABA (Gonzalez-Guzman et al., 2012), (Nishimura et al., 2010). The mimicking *pp2c* null-mutant showed elevated levels of abre.genes and exhibited ABA-insensitive characteristics since abre.genes concentrations with and without ABA were not significantly different (32310.3 µM and 32310.8 µM) (Fig. 4.5). Such a high concentration of mRNAs would most likely not accumulate in a real cell. Nevertheless, experimental data shows Arabidopsis null-mutants of *pp2cs* show a constitutive ABA response (Rubio et al., 2009). Mimicking *snrk2* and *abf* null-mutants had zero or near-zero abre.genes accumulation (Fig. 4.5) which is similar to experimental data on *snrk2.2/3/6* triple knockout mutants that were shown to be ABA insensitive and expression of ABA-induced genes was impaired (Fujii and Zhu, 2009), (Fujita et al., 2009). Similarly, a quadruple *abf/abre* mutant was found to have reduced ABA-induced gene expression (Yoshida et al., 2015). After this validation, we concluded that the transcription rate constant and feedback loops were important in creating a transient increase of the *ABRE* gene expression although the parameter values estimated may not truly present values in actual plants.
Figure 4.5. Mutant simulations show a similar outcome to actual mutated plants with respect to ABRE gene expression. Mutant simulations were made on the model by setting a parameter of a transcriptional rate constant for proteins PP2C, PYR, SnRK2, or ABF to zero separately for each mutational mimic. The simulations were then made with and without 100 µM ABA and the abre.gene concentration at each of the simulations' peak time points was determined and graphed. Highlighted in blue is data simulated with ABA and highlighted in orange is data simulated without ABA.

4.3.4 Predictions and generation of hypothesis

1. Feedback regulation of PP2C, but not PP2CA nor ABF, is sufficient for transient activation of the ABRE promoter.

After we confirmed the approximation of our model, we utilized the model to predict system behavior in the ABA signaling pathway. First, we predicted which feedback mechanism, PP2C (a protein that dephosphorylates SnRK2), PP2CA (a protein that dephosphorylates ABF-P), or ABF, or any combination of them is necessary for transient activation of the ABRE promoter. Namely, we removed the feedback regulations of PP2C, PP2CA, and ABF sequentially by setting a translation rate constant (ABA related) for each protein to zero. The analysis found that removing the PP2CA or ABF feedback retains the abre.genes transient expression (Fig. 4.6a & b) while the removal of the PP2C feedback loop only makes the accumulation of abre.gene be logarithmic (Fig. 

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4.6c). On removing both PP2Cs (PP2C and PP2CA), accumulation of abre.gene increased, and the logarithmic curve became steeper (Fig. 4.6d). This indicates that the negative feedback of PP2C (where it dephosphorylates SnRK2 kinases) is necessary for creating the transient activation of the ABRE promoter.

Figure 4.6. The feedback loop on PP2C is necessary for a transient activation of the ABRE promoter. Simulation output showing accumulation of abre.genes in the model without feedback loop(s) in ABF (a), PP2CA (b), PP2C (c) and both PP2C and PP2CA (d).
2. The translation of PP2C plays a significant role in the activation of the \textit{ABRE} promoter.

To understand important parameters with respect to the \textit{ABRE} promoter activation which is preceded by ABF-P accumulation, we conducted a sensitivity analysis of key parameters against the accumulation of abre.genes in the model. This determines which of the reactions or parameters, the accumulation of the abre.genes is most sensitive to. Namely, we compared parameters of dissociation rate constants in protein-protein or protein-promoter interactions, catalytic rate constants in enzymatic reactions, transcriptional rate constant in the \textit{ABRE} promoter, and translational rate constants in the feedback-regulated proteins (Table 4.2). The analysis found that while most of the selected parameters are equally sensitive, parameters related to ABA and PYR binding are least sensitive while the parameter related to the translation of PP2C had the highest sensitivity for accumulation of abre.genes (Fig. 4.7).

![Sensitivity scores against abre.gene](image)

**Figure 4.7.** Sensitivity scores against abre.gene. Parameters were analyzed on their sensitivity to the output of abre.gene. Descriptions of the parameters analyzed are listed in Table 4.2.
Table 4.2. Description of parameters used in the ABRE promoter activation sensitivity assay.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kr3</td>
<td>ABA and PYR binding</td>
</tr>
<tr>
<td>Kr4</td>
<td>PP2C and SnRK2 binding</td>
</tr>
<tr>
<td>Kr5</td>
<td>PP2C and SnRK2-P binding</td>
</tr>
<tr>
<td>Kr6</td>
<td>SnRK2 and MAP3K binding</td>
</tr>
<tr>
<td>Kf7</td>
<td>Phosphorylation of SnRK2 to SnRK2-P</td>
</tr>
<tr>
<td>Kr8</td>
<td>Snrk2-P and ABF binding</td>
</tr>
<tr>
<td>Kr9</td>
<td>PYR.ABA and PP2C binding</td>
</tr>
<tr>
<td>Kr10</td>
<td>PYR.ABA and PP2C.SnRK2 binding</td>
</tr>
<tr>
<td>Kr11</td>
<td>ABF-P and PP2CA binding</td>
</tr>
<tr>
<td>Kr12</td>
<td>ABF-P and ABRE binding</td>
</tr>
<tr>
<td>Kf13</td>
<td>Release of SnRK2 from ABA.PYR.PP2C. Snrk2 complex</td>
</tr>
<tr>
<td>Kf14</td>
<td>Dephosphorylation of SnRK2-P</td>
</tr>
<tr>
<td>Kf15</td>
<td>Phosphorylation of ABF</td>
</tr>
<tr>
<td>Kr16</td>
<td>Dephosphorylation of ABF-P</td>
</tr>
<tr>
<td>Kf17</td>
<td>Transcription of ABA-induced genes</td>
</tr>
<tr>
<td>Kf18</td>
<td>Translation of ABF</td>
</tr>
<tr>
<td>Kf19</td>
<td>Translation of PP2C</td>
</tr>
</tbody>
</table>

To further determine how the translational rate of PP2C affects the ABRE promoter activity, we analyzed how the parameter for translation of the PP2Cs (kf20) affects the accumulation of abre.genes. We found that the parameter affects the kinetics of abre.genes significantly. Namely, it affects not only the maximum concentration of abre.genes in a cell but
also the peak time point when the highest concentration of abre.genes is achieved (Fig. 4.8). As we increased the parameter value of the translational rate constant from the originally set value 4.5 hr\(^{-1}\) to 50 hr\(^{-1}\), concentrations of abre.genes were reduced, and the peak time point was earlier (Fig. 4.8). Learning that the accumulation of abre.genes is largely affected by the translation rate of PP2C, we wondered whether the translation rates are affected by ABA itself.

![Figure 4.8. An increase in the translation rate of PP2C is important in regulating ABRE gene expression. Parameter kf20 was adjusted from 4.5 hr\(^{-1}\) to 10, 20, and 50 hr\(^{-1}\), and the ABRE gene expression was simulated. Notice a level of accumulation and a peak time point of the accumulation are changed with a function of translational rate constant.](image)

To this end, we searched literature that study changes in translation rate in Arabidopsis plants. We found that while direct measurement of translational rate in eukaryotic cells has been studied only in yeast and animal cells (Weinberg et al., 2016), (Schwanhäusser et al., 2011), indirect measurement has been conducted in plants as well (Fujita et al., 2019). In an indirect measurement, which is known as ribosomal profiling, a ratio of ribosome-protected mRNA fragments over total mRNA expressed in cells is measured at a given time point. In theory, a higher ratio of ribosome-protected mRNA over total mRNA indicates a higher translational rate at the given time point.
A previously conducted study with a DNA microarray found that translational rates in all PP2Cs involved in the ABRE promoter activity (namely ABI1, ABI2, HAB1, PP2CA) are downregulated due to dehydration (Table 4.3), (Kawaguchi et al., 2004). Because a microarray used in the study does not contain a completed set of gene probes, a change in the translational rate of all ABFs involved in the ABRE promoter activity (namely ABF2, ABF3, and ABF4) is not conclusive. However, based on the available data (ABF3), we can predict that the translational rate of ABFs may be downregulated. This suggests that the translation rate in PP2C may indeed be downregulated during ABA signaling transduction.

On the other hand, a most recently conducted study with deep RNA-sequencing technology, in which all expressed mRNAs are measured, found that the translational rate in all ABFs involved in the ABRE promoter activity (ABF2, ABF3, and ABF4) are up-regulated while almost all PP2Cs (data for ABI2 is not available) involved are little changed upon exposure of exogenously added TOR inhibitor (Table 4.3), (Scarpin et al., 2020). The study concluded that the plant TOR specifically controls the translation of a set of mRNAs that possesses 5’ oligopyrimidine tract motifs (5’TOPs), which results in an alternation of translation in other genes as well (Scarpin et al., 2020).

Based on these two studies and the sensitive analysis of our model, we predicted that combinational exposure of ABA and a TOR inhibitor up-regulates the activity of the ABRE promoter. The rationale is as follows. First, upon ABA exposure, transcription of PP2Cs and ABFs are up-regulated due to the activation of the ABRE promoter (Wang et al., 2019). Second, however, the translational rate of PP2Cs and likely ABFs as well are downregulated by yet unknown mechanisms (Kawaguchi et al., 2004), resulting in diminishing the effect of up-regulation of the transcription of PP2Cs and ABFs (Scarpin et al., 2020). Third, by exposing a TOR inhibitor, the
translational rate of ABFs is increased while that of PP2Cs is not changed (Scarpin et al., 2020). As a result, by exposing ABA and a TOR inhibitor, the activity of the ABRE promoter increases.

To examine the prediction, we analyzed the ABRE promoter activity in the RD29A::LUC transgenic plants (Ishitani et al., 1997). As a control, we analyzed the 35S promoter activity in the CAMV35S::LUC transgenic plants (Rosin et al., 2008). The 35S promoter is a constitutive promoter that does not contain the ABRE cis-element and therefore its expression would not be induced with ABA. When the RD29A::LUC transgenic plants were exposed to rapamycin, luciferase activity was little alternated (Fig. 4.9). When the plant was exposed to ABA, luciferase activity was increased (Fig. 4.9). When both rapamycin and ABA were exposed to the plant, the luciferase activity was 4-fold higher than of ABA at the maximum time point (Fig. 4.9). This result suggested that our prediction agrees with the behavior of actual plants.

Table 4.3. Change in translational levels of PP2Cs and ABFs involved in the ABA signaling pathway found in previously published literature. Translational levels of respective mRNA were profiled using DNA microarrays on mRNA in polysomal complexes as a measure of the rate of translation in Arabidopsis plants exposed to dehydration stress (Kawaguchi et al., 2004). Change in the translation of mRNA species was also profiled using Riboseq/RNA seq data sets on the effect of TOR inhibitor Torin2 in Arabidopsis plants (Scarpin et al., 2020).

<table>
<thead>
<tr>
<th>mRNA species</th>
<th>Changes in relative translation rate with dehydration compared to control condition. (Kawaguchi et al., 2004)</th>
<th>Changes in relative translation rate in TOR inhibition, compared to a control condition (Scarpin et al., 2020)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABI1</td>
<td>0.92</td>
<td>1</td>
</tr>
<tr>
<td>ABI2</td>
<td>0.95</td>
<td>Data not available.</td>
</tr>
<tr>
<td>HAB1</td>
<td>0.80</td>
<td>0.92</td>
</tr>
<tr>
<td>PP2CA</td>
<td>0.98</td>
<td>1.13</td>
</tr>
<tr>
<td>ABF2</td>
<td>Data not available.</td>
<td>1.39</td>
</tr>
<tr>
<td>ABF3</td>
<td>0.97</td>
<td>1.32</td>
</tr>
<tr>
<td>ABF4</td>
<td>Data not available.</td>
<td>1.15</td>
</tr>
</tbody>
</table>
**Figure 4.9.** Combinational exposure of ABA and rapamycin increases the ABRE promoter activity. Normalized luminescence from *RD29A::LUC* and *CAMV35S::LUC* plants when exposed to 200 µM ABA only or 200 µM ABA + rapamycin. Data shown is from means of 3 independent replicates and error bars derived from standard error to the mean. Data was normalized against control DMSO treated plants.

**4.4 Discussion**

Here we presented a model of the ABA signaling pathway that leads to the activation of ABF with core components. The model was built based on quantitative data analyzed *in vitro* in the past. The model may be far from a real plant because the model assumes reactions occur only in a single cell but not in the entire plant. Subcellular localization of molecules within the cell is also ignored. In addition, the model assumes that other molecules in the cell are not involved in the ABA signaling pathway. Indeed, quantitative predictions in the model disagree with real plant data when the ABRE promoter activity in ABA-dependent responses and in gene knockout mutants was compared (Fig. 4.2 and 4.5). However, the output dynamics of the model with respect to the activity of the ABRE promoter agreed with the previously reported qualitative data in real plants such as phenotypes of the *RD29A* gene expression in gene knockout of the core components in the ABA signaling pathway (Fig. 4.5).
The model suggests that the feedback regulation (loop) in which expression of genes in PP2C is up-regulated upon ABA exposure, allows the transient up-regulation of the ABRE promoter. Without the feedback loop, the activity would be logarithmic and not show the transient increase (Fig 4.2e). The model also suggests that the translational rate for PP2C in the feedback loop is the most sensitive parameter for activation of the ABRE promoter (Fig. 4.7). We discovered that a high value of the translation rate constant reduces the ABRE promoter activity, but the activity peak reaches a maximum at an earlier time point (Fig. 4.8). This suggested that the translational rate constant in the feedback regulation would be one of the most key factors that fine tunes the kinetics of the ABRE promoter.

Our literature search determined that the translational rate of PP2Cs is down regulated upon dehydration (Table 4.3). This suggests that activity of the ABRE promoter is regulated by not only up-regulation of the gene expression but also down-regulation of the protein translation on PP2Cs and ABFs. We propose that these two opposite regulations, up-regulation of gene transcription and down-regulation of protein translation in PP2C would allow the ABRE promoter to determine expression levels and expression peak time. We showed that the activity of the ABRE promoter is indeed up-regulated by inhibiting the TOR activity that leads to the up-regulation of the translation rate of ABFs independent from ABA. (Fig. 4.9).

In the past, the accumulation of mRNA and post-translational modification of proteins were thought to define the activity of the ABRE promoter (Joo et al., 2021), (Nordin et al., 1993). However, our model and experimental data suggest that changes in translational rates would also largely determine the activity of the ABRE promoter. We, therefore, suggest that investigating the alteration of translational rates in proteins, typically PP2Cs, is the next frontier in the research field of the ABA signaling pathway.
We are aware that not only the translational rate but also the degradation rate of proteins, which are not investigated in this study, are important in the ABA signaling pathway (Ali et al., 2019), (Wu et al., 2016). We are also aware that it is not only ABFs but also other transcription factors that bind to the \textit{ABRE} cis element (Song et al., 2016). Hence, the activity of the \textit{ABRE} promoter does not depend only on ABF activities in actual plants while we assume only the activation of ABF activates the \textit{ABRE} promoter in the model.

Nonetheless, the model of the core components in the ABA signaling pathway presented in the model should help to understand the ABA signaling pathway more qualitatively and make novel hypotheses that are difficult to make by simply looking at conventional diagrams of a signaling pathway. An example is shown here is the importance of changes in translational rates in PP2C.
ABA and PA are important signaling molecules when a plant responds to drought, salinity, and cold. They are both synthesized when plants detect stress and their signaling pathways aid the plant in withstanding the stress. ABA a sesquiterpenoid that is synthesized from a C40 molecule through a series of different enzymes. On its synthesis it regulates different roles in the plant including seed embryo maturation, seed dormancy and responses to drought, cold, and salinity. The best studied ABA response is stomatal closure which leads to reduced transpiration allowing the plant to preserve water and therefore maintain a low water potential in the plants.

PA on the other hand exists as a structural phospholipid and synthesized in the ER or plastids as a precursor for other membrane phospholipids. As a structural phospholipid, PA constitutes about 1% of the total phospholipids in a plant. Signaling PA is synthesized transiently within minutes and is quickly hydrolyzed after transmission of the signal to maintain the low concentrations. Signaling PA has been linked to many plant responses including drought, salinity, cold, wounding and pathogen response. Different species of PA exist, and these are synthesized depending on the type of response. For example, PA species 34:1, 34:2, 34:3, 36:3, or 36:6 are synthesized as a response to ABA. Therefore, it is important to discriminate the species of PA formed in each of the different stress responses to be able to understand the different signaling pathways.

In my dissertation research we found out that although PA is involved in ABA-mediated stomatal closure, we could not determine that it regulated ABA-mediated R\textit{D29A} gene promoter activation. R\textit{D29A} gene is an ABA-induced gene that contains an \textit{ABRE} cis element on its promoter that is ABA regulated. The gene also contains additional cis elements that are regulated in response to drought, salinity and cold. Therefore, we cannot rule out that genes that are only
regulated by ABA i.e., they only contain the ABRE cis element e.g., RD29B and RAB18 might show a different response pattern. In addition, we were able to show the spatiotemporal formation of PA in vivo using fluorescent proteins. This PA biosensor enabled the determination of PA formation in minutes and its localization since the fluorescent proteins were localized on both the plasma and internal membranes. On addition of either ABA or NaCl, there was an increase in PA formation on the plasma membrane, but the results in the internal membranes were conflicting. While ABA enhanced PA synthesis on the internal membranes the same was not observed with NaCl since the control and treated samples were not significantly different. The PA biosensor is a novel idea for determining PA synthesis both on the plasma and internal membranes. However, further optimization of the biosensor is required.

In my final topic on determining the ABA signaling dynamics, we determined that translation of PP2C phosphatases, which are negative regulators of the ABA signaling pathway is important in determining the kinetics of the ABA response. When the rates of translation of PP2C were enhanced ABA-mediated gene expression was reduced and the peak time point of expression was at an earlier time point. Though there is a lot of focus on transcription and post-translational regulation of proteins involved in the ABA signaling pathway, we propose that the rate of translation is equally important in regulation of the ABA signaling dynamics.

Our findings are important advancements in ABA and PA signaling responses that will help gain a better understanding of these pathways. Understanding these signaling responses would be an important step in deciphering how plants respond to drought. This is crucial in determining ways of mitigating drought effects on plants. Further research should be conducted on why PA does not play a role in ABA-mediated gene expression but is involved in ABA-mediated stomatal closure. However, based on our results we can propose that both PA and ABA are important in the
response of plants to drought and therefore use of these molecules could be applied to crop plants to enhance their drought resistance.

Based on the dissertation research outcomes I propose that the use of exogenously applied PA and ABA could be used in crop plants to enhance their drought response. On one hand, PA promotes stomatal closure but not ABA-induced gene expression while ABA promotes both stomatal closure and ABA-induced gene expression which alters the cellular concentrations of proteins important in drought response and this changes the cellular dynamics in other cells other than the guard cells. Therefore, use of these two molecules could be used alternately depending on the expected outcome in the plant i.e., stomatal closure only to restrict transpiration or both stomatal closure and drought induced gene expression within the plant. This would alleviate the creation and use of transgenic plants that are drought resistant which might take a long time to develop in addition to altering the biodiversity on their release to the natural environment.
APPENDIX A.
RAT14 cDNA SEQUENCE

```
NcoI
BamHI
CACTATAGGGCGAATTGAAGGAAAGCCGTCAGGGCCGATGATCCATGCTGAAGCAAGAG
1  ---------------------------------------------------------------+
GTGATATCCCGCTTAAACTTCTTTCCGGCAGTTCCGGCGGCATTGACCTTACCTGCCTTCTC

CGAGGGGATTCATCGAGATGTCGAGAGGGGCGAGGCGGACCCCTACGAGGACCCAGACCG
61 ---------------------------------------------------------------+
GCTCTCCAGCTAGGTCTCAGTACGAACTGCTCCCGCTCCCGCTGCCCGAGGATGCTCCCGTGCTGGGC

CCACGAGTTTCGAGATCGAGGGCGGCGAAGGGGCAGCGCCGACCCCTACGAGGACCCAGACCG
121 ---------------------------------------------------------------+
GGTGCTCAAGCTCTAGCTCCCGCTCCCGCTCCCGGGAGGAGACCTGTAGGACAGGGGGT

BstEII
CAAGCTGAACTCAGAACAAGGCGCCCTCAGGGACATCTGCTTGCCCACA
181 ---------------------------------------------------------------+
GTTGACTTCCACTCTGCTCCGGGCTCCCGAGGAGGCTCCTCCTGGCTGGCG

BsiWI               AatII
GATCATGTACGGCTCCAAGGCGTACGTGAAGCACCCCGCCGACGTCCCCGATTACATGAA
241 ---------------------------------------------------------------+
CTAGTACATGCCGAGTCTCCCGCATGCACTTCTCGTGAGGCCTGAGGCTAGGGCTAATGTACTT

GCTGTCCCTCCCGAGGCTCAGTGGGAGGCTGATGCCACCTCGAGGACGGCGGTCT
301 ---------------------------------------------------------------+
CGACAGGAAGGGCTCCGGAAGTTCACCCTCCGCTGCACTACGTGAAAGCTCTCTCCGCGCAGA

BstEII                  PstI
GTTGACCGTGACCTCGAGACCCGACCCTCTCGGAGGACGTACCATCAAAGGTGAAGAT
361 ---------------------------------------------------------------+
CCACTGGGACTGCTGCTGGAGGACGTCCGGGCTGAGCTAGAGTGTTCCACTTCTA

BbsI
GGCGGGCAACCAACCTCCTCCCCCGGCAGCCGGCGGATATCGAGAAGAGACCTTGAGCTGGGA
421 ---------------------------------------------------------------+
CGCGCCGTGTGAGGGGCGGCTGCCCAGGCTTACGTCTTCTCTCTGGAAACCAGCACC

NarI
BbsI                 KasI
```
APPENDIX B.

B3T14 cDNA SEQUENCE

NcoI
BamHI

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1 -----------------------------------------------
GTGATATCCCGCTTAATCTCTCTTCCGCCAGGTCCGGGCTACCTAGTTACCGACTCTGCTTCT

BsaI

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121 -----------------------------------------------
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BstEII

CAAGCTGAGGTTGCAACAGGCTACGGGTACCCAGGACTCCTCCCTGCCAGTCTGGGACTTAAGAA

181 -----------------------------------------------
GGTGACCGTTACCAGGACTCCTCCCTGCCAGTCTGGGACTTAAGAA

BsiWI

GTTCATGTACGGCTCCGAGGCGTACGTGAGGCACCCCGCCGACATCCCCGATTACAAGAA

241 -----------------------------------------------
CGACGGGAAGGGCTCCCGAAGTCCTCGCACCTCGCTGTTGGGCTGGGCTTAGGGCTAGTGTTCTT

BstBI

GCTGCCCTTCCCCGGAGGGCTTCAAGTGGGAGCGCGTGATGAACTTCGAAGACGGCGGTCT

301 -----------------------------------------------
CGACGGGAAGGGCTCCCGAAGTCCTCGCACCTCGCTGTTGGGCTGGGCTTAGGGCTAGTGTTCT

BstEII

PstI

GTGGACCGTTACCAGGACTCCTCCCTGCCAGGAAGGCCACGCTGATCTGCAAGGTGAAGAT

361 -----------------------------------------------
CCACTGGCAATGGGTCCTGAGGAGGGACGTCCCGCTGCGACTAGGTCTCCACTCTTCTTGCGC

PflMI

BbsI NcoI StuI

GCGCGGACCCACTCTCCCTCCCCGGAGGCCACGCTGATCTGCAAGGTGAAGAT

421 -----------------------------------------------
CGCGCCGTGTTGAAGGGGGGCTGCCGGGCATTACGTCTCTCTCTGTTACCAGCCCT
BbsI
GGCCTCCACCGGAGATGCTGTACCAGCCAGGACGGGCGTGCTGAAGGGCCATAGCTATCAGGCG

PflMI              BsaI
CCTGAAGCTGAAGGACGGCGGCCACTACCTGGTGGGTTGAGACCATCTACATGGCCAA

NheI                     HindIII
CATGGACGAGCGGTACAAGAGCACGGCTAGCACGGAGATAGATAAGACTAAAGCTTTTGG

BbsI  AccI             PmeI
TCGAGGGTCCTCTGCTACAGTAGCTGCTGTATGTAAACAAATTTGGCAAGCATTTGAAGGA

XhoI                          SacI
TAAATCTTTTGAGCATTGGCTGCAAGCACCCACCACCACCACGTGAGAGCTCCTGGG

CCTCATGGGCCCCTTTCACCTAGCCCGCCTTTCCAG

GGAGTACCAGGAAAGGAGCGGGCAGGTCGACT
APPENDIX C.
GA cDNA SEQUENCE

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<tr>
<td>BssHII</td>
<td>CGAATTGCGGAAGGCCGTCAAGGCCACGTGTCCTTGCCAGGCGCCAGGGATCCATGGT</td>
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<td>AscI</td>
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<tr>
<td>NcoI</td>
<td>GAGCAAGAGCGAGGAGGTGTCATCAAGAGTTCATGCGCTTCAAGGTGCGCTTGGAGGGCTC</td>
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<tr>
<td>BamHI</td>
<td>CTCGTTCTCGCTCTCCAGTATGTTTCTCAAGTACGCGAAGTTCCACGGAAGTCTCCCGAG</td>
</tr>
<tr>
<td>BstEII</td>
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</tr>
<tr>
<td>BclI</td>
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</tr>
<tr>
<td>AatII</td>
<td>CAGGGGAGACTAGTACATGCAGGGGCTTTCTACATGCACTTCGGGCGGCTGCAGG</td>
</tr>
<tr>
<td>PstI</td>
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<tr>
<td>BstEII</td>
<td>CGGCGGTCTGGTGACCGGAACACAGGTGTACCTCCTGCCAGGACGCTGATCTACAA</td>
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<tr>
<td>BbsI</td>
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<td>SacI</td>
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    NarI
    KasI

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541  --------------------------------------------------
    GGACCCCGCGGACTTCTGACTTCTCGCCTCCGGACGTTGGACCCCTAGTTGCTGTAGAT

CATGGCCAAGAAGCCGCTGCAACTGCCCCGCTACTACTCTGTGGACACCAAGCTGGACAT
601  --------------------------------------------------
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    PflMI

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661  --------------------------------------------------
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    XhoI   PacI

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GAGCACAAGACTGCGCTCATGGGCCTTCCGCCTCAGCTACG
781  --------------------------------------------------
    CTCGTGTTCTGACCAGGAGTACCCCGGAGGCGAGTGACG
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(2003a) Transient expression vector pBI221, complete sequence.

(2003b) Binary vector pBI121, complete sequence.
VITA
Ruth Wanjiru Ndathe was born and raised in Kenya. As a young girl growing in rural Kenya, she dared to dream. She dreamt one day that she would be a renowned scientist providing solutions to global problems. Therefore, on joining college she chose to pursue a Bachelors in Education Science at Kenyatta university and proceeded to teach high school biology and chemistry before joining graduate school at Louisiana state university. She anticipates graduating in December of 2021 with a Ph.D. in Biochemistry where her dissertation research focused on drought signaling pathways in plants.