Crosstalk Between Ethylene and Cytokinin in *Arabidopsis thaliana*

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By

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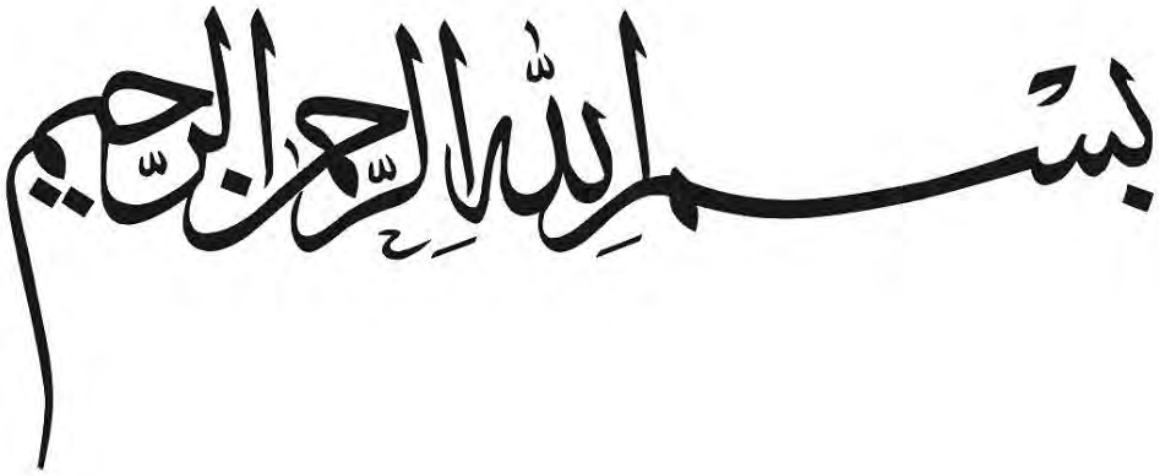

"In (or with) the name of Allah, the Beneficent, the Merciful"

Dedicated

To

My Beloved Husband and My Parents

DECLARATION

I hereby declare that the work presented in the following thesis is my effort and that this thesis is my composition. No part of the thesis has been previously presented for any other degree.

Aleena Ramzan

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All the praises are for my Lord, **Allah** (SWT), the most beneficent and merciful. He blessed me with all the golden opportunities, made all the required circumstances, and assigned me the best people to help me accomplish my goals. Peace and blessings of Almighty Allah (SWT) be upon His Prophet **Muhammad** (P.B.U.H) who exhorted his followers to seek knowledge from cradle to grave.

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May Almighty Allah shower His blessings and prosperity on all those who assisted me in any way during this research project.

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List of Abbreviations

ACC	1-aminocyclopropane-1-carboxylic acid
ACO	ACC Oxidase
ACS AACSCSCS	1-Aminocyclopropane -1-Carboxylic Acid Synthase
ARF	Auxin Response Factors
ARR	Arabidopsis Response Regulator
AHP1	Arabidopsis Histidine Phosphotransfer
AHK	Arabidopsis Histidine Kinase
ARGOS	Auxin Regulated Gene Involved in Organ Size
AVG	Aminoethoxyvinylglycine
BT	Beta tubulin
BA	Benzylaminopurine
CTR1	Constitutive Triple Response1
CMT	Cortical Microtubules
CKX	Cytokinin Oxidase
CHASE	Cyclases/Histidine Kinases Associated Sensor Extracellular)
DZ	Differentiation Zone
DMSO	Dimethyl Sulfoxide
EDTA	Ethylenediaminetetraacetic acid
EIN2	Ethylene Insensitive 2

EIN4	Ethylene Insensitive 4
EMB	Ethylene Binding Domain
SAM	S-Adenyl-methionine
SHY2	Shorthypocotyl 2
ER	Endoplasmic Reticulum
ERF1	Ethylene Response Factor1
ERF2	Ethylene Response Factor 2
ERS1	Ethylene Response Sensor 1
ERS2	Ethylene Response Sensor 2
ETR1	Ethylene Response 1
ETR2	Ethylene Response 2
EZ	Elongation Zone
GOF	Gain of Function
IAA	Indole-3-Acetic Acid
KMD	Kiss Me Deadly
LOF	Loss of Function
LOG	Lonely Guy
MCP	MethylCycloPropene
MZ	Meristematic Zone
PCD	Programmed Cell Death
PLT	Plethora

QC	Quiescent Centre
RAM	Root Apical Meristem
SCF	Skp, Cullin, F-box Containing Complex
XTH	Xyloglucan endotransglycolases/hydrolases

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Summary

Plant hormones control the growth and developmental patterns of plants. They interact with each other synergistically or antagonistically in response to external stimuli. Understanding the interactions of plant hormones is very important for the betterment of plant growth. This thesis provides information about plant hormones, particularly ethylene. This thesis is divided into two parts, i-e., Part A and Part B. **Part A** describes ethylene's role in inhibiting the root apical meristem. Ethylene is already known to inhibit the elongation of cells in the primary root. Our study finds that it inhibits the proliferation of cells in the meristem. Ethylene reduces the number of cells in the cortex layer of the root apical meristem. Ethylene-insensitive mutants do not show a decrease in the number of cells of the cortex layer as observed in the wild type. Our real-time data shows the cell cycle arrest at the root apical meristem by the induction of the ICK gene in response to ethylene. Inhibition of proliferation at root apical meristem is well studied in Auxin – Cytokinin interaction. Induction of Shy2 by cytokinin inhibits the proliferation. Our real-time data finds that ethylene induces Shy2 in the same manner as cytokinin to inhibit proliferation at root apical meristem. Our study indicates the interaction of ethylene and cytokinin as ethylene inhibits proliferation in the same manner as cytokinin in the meristem. We assume that ethylene recruits ARRs (*Arabidopsis* Response Regulators) of cytokinin signaling via the histidine kinase domain of ETR1. Interaction of ARRs with EIN3 (transcription factor of ethylene) controls the expression of ethylene response genes. We used pharmacological agent 1-MCP to recover the reduced size of meristem caused by ethylene in wild-type *Arabidopsis* seedlings. **Part B** of the thesis describes the ripening delay in tomatoes by 1-MCP. We described the benefits of 1-MCP in the agriculture industry. As ethylene is responsible for the ripening of fruits and vegetables 1-MCP can prolong the ripening by inhibiting ethylene. 1-MCP delayed the ripening of tomatoes and prolonged the storage period of tomatoes. Physiological parameters like color, firmness, and weight were maintained with 1-MCP for a longer period. Ripening-related genes RIN, CNR, NOR, and PSY were downregulated in response to 1-MCP.

Abstract

Plant hormones regulate the growth and development of plants throughout their life. Plants change their growth pattern to adjust according to the external environment. This change is controlled by the crosstalk of different hormones present in the plants. Ethylene is one of the most important hormones present in plants. It controls several developmental processes in plants that include senescence, abscission of leaves, ripening of fruits, defense against pathogenic attack, and stress conditions. It regulates the growth patterns of plants by interacting with other hormones. Crosstalk of hormones in the development of the root system of plants is critical. The development of the primary root greatly depends on the regulation of the proliferation of cells and the elongation/differentiation of cells in the root. Ethylene is known to inhibit the elongation of cells in the roots. Our study finds the role of ethylene in inhibiting the proliferation of cells in the root meristem. Ethylene reduces the number of cells in the cortex layer of the root apical meristem. Wild-type *Arabidopsis thaliana* shows a reduced meristem in ethylene response. Insensitive mutants of ethylene show no reduction in the root meristem. Real-time data shows that the reduction in root meristem by ethylene is due to shy2. Shy2 inhibits RAM in response to ethylene exactly in the same manner as it does in response to cytokinin. Ethylene and cytokinin both inhibit the proliferation of cells in the root apical meristem. Our study indicates the interaction of ethylene and cytokinin and the recruitment of ARR by ethylene via the histidine kinase receptor of ETR1. Concurrent treatment of ethylene and cytokinin induced a subtractive and additive effect on the expression of cytokinin and ethylene-induced genes in wild type. Based on this, we predict that ARRs regulate a subset of ethylene responses, doing so through interaction with the EIN3 family of transcription factors. The use of pharmacological agent 1-MCP recovered the reduced size of the meristem indicating the role of ethylene in inhibiting root apical meristem. Our study also provides the agricultural benefits of 1-MCP. 1-MCP delayed the ripening of tomatoes and prolonged the storage period of tomatoes. Physiological parameters like color, firmness, and weight were maintained with 1-MCP for a longer period. Ripening-related genes RIN, CNR, NOR, and PSY were downregulated in response to 1-MCP. 1-MCP treatment improves the quality of tomatoes for longer periods and is useful for longer storage and transportation. The ripening effect of ethylene can be delayed by the use of 1-MCP.

INTRODUCTION

1.1 Plant Hormones

Plant hormones are smaller molecules that are synthesized in plants from different metabolic pathways. These small molecules are present at very low concentrations in plants. The site of action for these hormones is either local, where they are synthesized or they can also travel long distances in plants to perform their action. Plant hormones perform various essential functions in plants. They are released in response to several biotic and abiotic stress environments. They are also responsible for the growth and development of plants (Santner et al., 2009). Exposure of plants to a variety of environmental stresses throughout their life cycle utilizes some special mechanisms to overcome the changes in the external stimuli for their proper growth and development. Hormones in the plant have special actions in growth and development. Until now ten small structurally distinct molecules have been identified to regulate plant growth and are defensive against harmful attacks known as plant hormones (Verma et al., 2016).

Phytohormones were identified during the first half of the twentieth century, and are termed as classical hormones comprising gibberellin, cytokinin, abscisic acid, auxin, and ethylene (J. Davies, 1995). Other compounds such as nitric oxide, jasmonate, salicylic acid, strigolactones, and brassinosteroids are also characterized as phytohormones (Gomez-Roldan et al., 2008, Vert et al., 2005). With the advancement of molecular biology research in recent times, several plant hormone receptors have already been recognized and characterized (Wang et al., 2015).

1.2 Hormonal Regulation in Plant Growth

Plant hormones act by forming a signal network through signal transduction pathways. Different hormones often interact with each other to control a developmental process. Such interactions are called crosstalk of hormones. Hormone signal transduction pathways are either phosphorelay pathways or they act by activating a second messenger (Mohamed, 2017). Sometimes a single hormone can regulate distinct cellular and developmental activities and sometimes multiple hormones are required to regulate a single cellular process. Several examples of hormonal regulation are

ethylene ripening the fruits, auxin & cytokinin regulating the cell cycle, seed germination induction and elongation of stems by GA, and seed dormancy maintenance by ABA. In the past one could only easily determine the effects of any hormone by applying it exogenously on plants or selected tissues. But today, the availability of mutants of several hormones and some response molecules helped in revealing the roles of the phytohormones in plants more clearly and broadly (Himmelbach et al., 2003)

Understanding the molecular mechanisms of plant hormones and their ultimate response in plant growth can help in the improvement of plants. Plant hormones and their functions can be manipulated resulting in better phenotypic traits (Gray, 2004). *Arabidopsis thaliana* has always been a model of choice for elucidating molecular mechanisms of phytohormones. Mutations in the genes have helped to confer the function of those genes in response to the hormone applied (Gray, 2004).

1.3 Ethylene a Plant Hormone

Ethylene being the first identified gaseous plant hormone is the simplest alkene C_2H_4 (Burg, 1973, Kepinski and Leyser, 2003). The significant role of ethylene comes into play at the very beginning of plant life. Ethylene helps the seedling to emerge out of the soil by pushing it out from the soil and forming an apical hook for the protection of delicate hypocotyl from any damage when it pushes itself out of the soil. The role of ethylene remains important from the start of the life cycle till the senescence of organs and abscission of leaves. It promotes seed germination, controls the growth of seedlings, regulates cell elongation and division of cells in the meristem, and rises in stress (Zhu et al., 2006). It also has a very significant role in the development of flower and sex determination, ripens the fruits, and then organ senescence is regulated at the end stages of the plant (Lin et al., 2009).

Starch is converted to simple sugar by ethylene during the process of ripening which leads to the softening of fruits and causes a color change in the skin of the fruit. (Abeles et al., 2012). Both exogenous and endogenous ethylene levels are responsible for stimulating ripening in plants (Bradford, 2008). Ethylene is considered to be a stress hormone as its levels rise in plants due to environmental stress like temperature, water, and nutrient stress as well as developmental stress that includes wound or attack by a pathogen. (Iqbal et al., 2013). A wide range of ethylene concentrations can be sensed

by *A. thaliana* seedlings and it can respond to a tremendous concentration (Binder et al., 2004).

1.4 Emergence of Ethylene Signaling in Plants

Ethylene receptors are widely studied and ethylene binding to its receptors in all land plants has been identified (Wang et al., 2006). Receiver domains of all the receptors of ethylene are conserved between the species of land plants and algae (Wang et al., 2015). Ethylene receptors are believed to be originated from cyanobacteria in ancient times because the homolog of the receptor gene is present in cyanobacteria (Wang et al., 2006). Acquisition of the ethylene receptor gene from cyanobacteria could have occurred through the process of endosymbiosis for 1 billion years (Sanderson et al., 2004). Cyanobacteria consists of a homolog of the ethylene receptor gene but it does not contain other signaling components of the ethylene pathway or they are not conserved in cyanobacteria (Ju and Chang, 2015).

The evolution of freshwater algae, the charophyte began with the evolution and function of the ethylene hormone (Ju and Chang, 2015, Van de Poel et al., 2015), which evolved in land plants about 450 million years ago (Sanderson et al., 2004). The evolutionary analysis and the studies of comparative genomics suggest that the development of the ethylene signaling pathway began after the evolutionary splitting of vascular and moss plants (Wang et al., 2018). Submergence responses mediated by ethylene were reported in mosses (Yasumura et al., 2012). Similarly, several reports suggest that the ethylene pathway originated, while the occurrence of angiosperms predates when monocots were diverging (Wang et al., 2015).

1.5 Ethylene Biosynthesis

Plants control the regulation of ethylene by producing endogenous ethylene according to their needs (Burstenbinder and Sauter, 2012). Production of ethylene occurs in every part of the higher plant with most actively produced in the meristematic regions. Endogenous levels of ethylene rise in plants in conditions such as senescence or different stress conditions where plants need ethylene to play its role (White et al., 1992).

In 1964, the discovery of ethylene biosynthesis took place with methionine found to be its precursor (Lieberman and Mapson, 1964). The Steps of ethylene biosynthesis from methionine stayed unclear for 15 years till 1979 when ACC (Aminoacyl

cyclopropane) was discovered as a midway component in the synthetic path of ethylene from methionine (Adams and Yang, 1979). The first step of the biosynthesis of ethylene involves the amino acid methionine. Ethylene biosynthesis takes place in three steps as follows:

- First biosynthetic step of ethylene is carried out by SAM (S-Adenyl-methionine) synthetase by converting methionine to its activated form S-Adenyl-methionine
- S-Adenyl-methionine is then converted to ACC by ACC synthase.
- Third step in ethylene biosynthesis is a rate-limiting step in which ACC is oxidized to ethylene, a reaction carried out by ACC oxidase. By product of MTA (5'Methyleadenosine) is produced in the last step.

MTA is used to regenerate methionine in the Yang cycle, recycling of MTA takes place to produce methionine to avoid its production to stop and keep going (Adams and Yang, 1979).

Production of endogenous levels of ethylene is generally low in plants and is usually increased by developmental or external signals (Chen et al., 2005). While higher ACC synthase activity is reported in ethylene over-producer mutants (eto) (Woeste et al., 1999). Levels of ACC synthase in plants correlate to the ethylene levels present in plants (Chae et al., 2003).

1.6 Components of Ethylene Signal Pathway

Genetic studies and molecular methodologies used in the early 1990s helped in the isolation of components present in the ethylene signal pathway. Isolated components consist of five ethylene receptors, CTR1 (Constitutive Triple Response) (a Ser-Thr kinase) acts as a negative regulator (Huang et al., 2003), EIN2 (ETHYLENE INSENSITIVE2), N-ramp like transmembrane, and EIN3/EIL1 (ETHYLENE INSENSITIVE3/ETHYLENE INSENSITIVE3) like that belong to a transcription factor family (Alonso et al., 1999). Ethylene signal transduction operates in a straight line according to the model (Fig 1.1).

Ethylene receptors and CTR1 negatively regulate signal pathways (Hua et al., 1995, Qu and Schaller, 2004, Wang et al., 2003), followed by EIN2 & EIN3/EIL1 which positively regulate the signaling. Ethylene binding to its receptor inhibits the negative regulation by CTR1 and releases EIN2 to positively regulate the signaling pathway

(Shakeel et al., 2013). In ethylene's absence, signal transduction is hindered by the negative regulation of the receptors. Receptors interact with the N-terminal domain of CTR1 via its histidine kinase domain. This interaction is a protein-protein interaction to promote the kinase activity of CTR. It Phosphorylates the C-terminal domain of the EIN2 (positive regulator) to prevent its movement to the nucleus and hence represses the ethylene signal response. While, in the nucleus, degradation of EIN3 (transcription factor) occurs through the SCF (Skp1, Cullins, F-box proteins) E3 ligase complex consisting of EBF1 and EBF2 (EIN3-BINDING F BOX PROTEINs) protein of F-box whereas EIN2 degradation in the membrane is done by the SCF complex consisting of ETP1 and ETP2 (EIN2 targeting protein 1 and 2) (Ju et al., 2012).

On binding ethylene to its receptor, the ethylene receptors and downstream CTR1 become deactivated. This causes EIN2 to become active and transduce a signal for ethylene response. Down-regulation of ETP1 and ETP2 increases the amounts of EIN2 in response to ethylene. CTR1 inactivation leads to EIN2 dephosphorylation and its cleavage; so, the C-terminal domain of EIN2 leads it to the nucleus and N-terminal is connected to the membrane (Ji and Guo, 2013). EIN2 with its C-terminal moves to the nucleus that results in EIN3 post-translational stabilization (Guo and Ecker, 2003, Chao et al., 1997). Then EIN3 binds to ethylene response gene promoters like ERF1 and ERF2 (ETHYLENE RESPONSIVE FACTOR) to initiate the ethylene response (Kendrick and Chang, 2008, Solano et al., 1998).

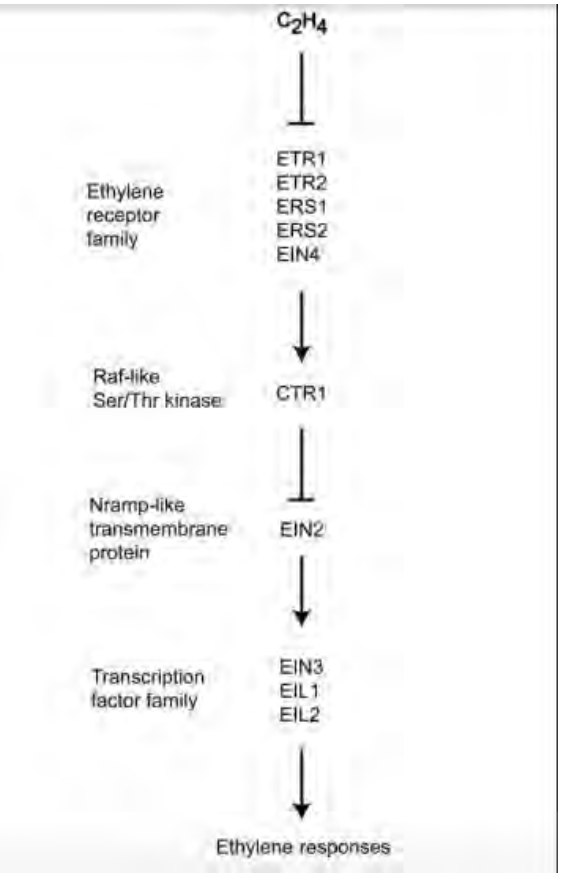

Figure 1.1: Linear Model Representing the Events of Ethylene Pathway. The Ethylene signaling pathway includes five ethylene receptors and a kinase-like CTR1 act in negative regulation while EIN2 and EIN3 positively regulate the ethylene signaling (Shakeel et al., 2013).

1.7 Triple Response Phenotype

Etiolated seedlings of *Arabidopsis* show a characteristic feature known as a triple response when grown with ethylene. Mutations causing insensitivity to ethylene do not show a triple response when treated with ethylene. The triple response was then used for the screening of ethylene mutants in *Arabidopsis* (Crocker, 1932).

The triple response is a phenotype and is named triple response due to the following three phenotypes occurring together in etiolated seedlings in response to ethylene.

- Formation of exaggerated apical hook
- Root elongation is inhibited

- Shortening and thickening of hypocotyl

Etiolated seedlings grown in the air do not show triple response. Seedlings also do not show a triple response phenotype in response to any hormone other than ethylene. This response is highly specific to ethylene and is used for screening mutants of ethylene (Schaller and Kieber, 2002).

1.8 Mutagenesis

In 1988 mutants were created in *Arabidopsis* by treating them with Ethyl methanesulfonate (EMS), Diepoxybutane, and X-ray. First ethylene mutant was reported when ethylene insensitivity was observed in 3 out of 75000 progenies (M2) of plants (M1) treated with EMS to induce mutations (Bleecker et al., 1988), ethylene insensitivity is characterized when the triple response is not shown in ethylene's presence. *etr1-1* was one of those mutants.

Different mutations in ethylene response genes respond differently and are categorized as follows:

- Ethylene Insensitive: Triple response is not observed when ethylene is present.
- Tissue Specific: Insensitivity to ethylene-induced triple response is observed in only specific tissue.
- Constitutive Response: This shows the response of ethylene when ethylene is absent.
- Over Producers: Shows triple response when ethylene is absent but inhibitors of ethylene biosynthesis rescue phenotype.

Identification of ethylene mutants helped in elucidating ethylene signal transduction. Mutations identified the order of signaling components and inferred that the ethylene signaling pathway is linear. Receptors of the signaling pathway were identified as early components as mutations in these genes affected all aspects of ethylene response. ETR1(ETHYLENE RECEPTOR1) and EIN4 (ETHYLENE INSENSITIVE4) were identified as early components and placed above CTR1 and EIN2 was placed below CTR1 in the signal pathway (Roman et al., 1995). The nature of mutation either loss of function mutation or gain of function mutation helped in determining if the component positively regulates the ethylene signaling pathway or negatively.

a. Mutants with a Loss of Function mutation:

Different mutations occurring in ethylene receptors demonstrated that receptors functionally overlap with each other. LOF (Loss of function mutation) in a single receptor showed a minor response whereas double (mutation in two receptors), triple (mutation in three receptors), and quadruple (mutation in four receptors) exhibit a more prominent response phenotype, presenting a phenotype of constitutive response of ethylene (Hua et al., 1998), and identified that receptors negatively regulate ethylene signaling. Several studies reported that due to functional overlap in receptors single LOF mutants do not necessarily show a noticeable phenotype due to compensation of this loss of function in a single receptor by other receptors (Rai, 2016). The functional overlap in ethylene receptors was also found in tomatoes (Tieman et al., 2000). Nevertheless, LOF mutations occurring simultaneously in three of any five receptors of ethylene are strong enough to induce constitutive responses (Hua and Meyerowitz, 1998).

b. Gain of Function mutants:

Gain of function mutation in one of the five receptors of ethylene causes insensitivity to ethylene, suggesting that it negatively regulates the pathway. A receptor with a gain of function (GOF) mutation has altered the N-terminal region with a change in amino acid that causes plants to be ethylene insensitive (Sato-Nara et al., 1999). The N-terminal region of the receptor contains the binding site of ethylene, so mutation there causes a defect in ethylene binding which results in insensitivity to ethylene (Hall et al., 2000).

Table 1.1: Mutants of Ethylene Signaling Pathway isolated in *Arabidopsis thaliana*

S. No.	Mutant	Specification	Gene Product	References
1.	etr1-1	(Gain of Function) GOF	Ethylene Receptor	(Bleecker et al., 1988, Chang et al., 1993)
2.	etr1-9	(Loss of Function) LOF	Ethylene Receptor	(Qu et al., 2007)
3.	ers1-3	LOF	Ethylene Receptor	(Qu et al., 2007)
4.	etr1-9/ers1-3	LOF	Ethylene Receptor	(Qu et al., 2007)
5.	etr2-3/ein4/ers2-3	LOF	Ethylene Receptor	(Hu and Meyerowitz, 1998)
6.	ctr1-2	LOF	Ser/Thr kinase	(Zhao et al., 2002)
7.	ein2-1 & ein2-50	LOF	Nramp like Protein	(Roman et al., 1995; Alonso et al., 1999)
8.	ers2-3	LOF	Ethylene Receptor	(Hua and Meyerowitz, 1998)
9.	ein3/eil1	LOF	Transcription Factor	(Qu et al., 2007)

1.9 Ethylene Receptors

Ethylene receptors in plants have a histidine kinase domain similar to the histidine kinase domain of a two-component system found in prokaryotes. Most of the research regarding ethylene receptors is done in *Arabidopsis thaliana*, consisting of five

ethylene receptors ETR1(ETHYLENE RESPONSE1), ETR2 (ETHYLENE RESPONSE 2), EIN4 (ETHYLENE INSENSITIVE 4), ERS1 (ETHYLENE RESPONSE SENSOR 1), and ERS2 (ETHYLENE RESPONSE SENSOR 2) (Rodriguez et al., 1999, Schaller et al., 2011).

Ethylene receptors are present in the endoplasmic reticulum membrane as homodimers. Dimerization of receptors occurs by disulfide bonds between Cys residues present in the N-terminal transmembrane domain present in ER and Golgi (Mayerhofer et al., 2015, Müller-Dieckmann et al., 1999, Schaller and Bleecker, 1995). Ethylene receptors consist of an ER transmembrane ethylene binding domain towards the lumen of ER while signal output domains are located toward the cytosol (Chen et al., 2002). The receptors of ethylene are present as large protein complexes (Chen et al., 2010), these protein complexes interact with each other and other members of a pathway like CTR1 via protein-protein interactions (Shakeel et al., 2015).

1.10 Structural Features of Ethylene Receptors

Receptors of ethylene consist of a histidine kinase domain similar to the two-component system. Ethylene receptors have a hydrophobic transmembrane N-terminus domain that binds to ethylene called ethylene binding domains EBD (Schaller and Bleecker, 1995). N- terminal region consists of three conserved transmembrane domains that locate the receptor within the ER membrane (Ma et al., 2006). Next to N-terminal EBD is present a GAF (cGMP-specific phosphodiesterases, adenylyl cyclases, and FhlA) domain used in protein-protein interactions. The interaction of GAF-GAF domains can cause the dimerization of receptors (Grefen et al., 2008). After the GAF domain, the histidine kinase domain is present which is similar to the two-component system, next to a histidine kinase is a receiver domain (Gallie, 2015), which plays an important role in recovering the growth response of ethylene.

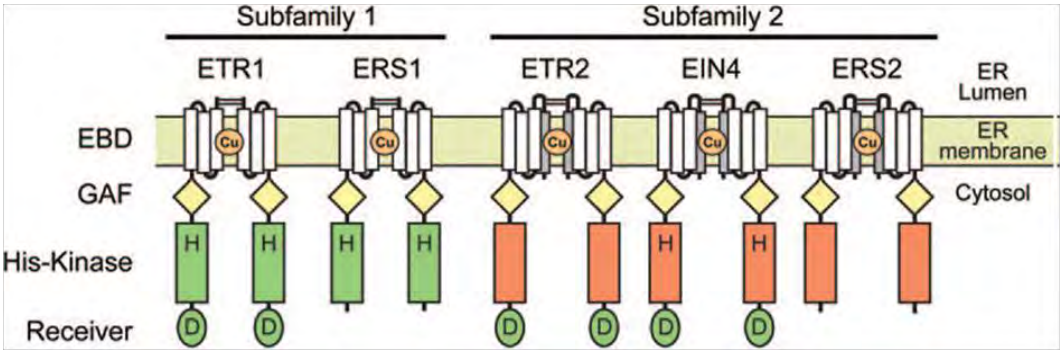

Figure 1.2: Structural Presentation of Ethylene Receptors Present in *Arabidopsis*. Structural features of five ethylene receptors and their division into two subfamilies based on the structural features and phylogenetic analysis. Three white rectangles represent the ethylene binding transmembrane domain with one additional grey rectangle in subfamily two containing a signal sequence. The GAF domain is represented as yellow diamonds. Green rectangles represent the conserved histidine kinase domain in subfamily one and diverged domain is present in red in subfamily two. Green ovals represent the receiver domain with conserved amino acids (Shakeel et al., 2013)

1.11 Ethylene in Plant Growth Regulation

1.11.1 Cell Division

Ethylene in sub-epidermal layers promotes cell division during the formation of apical hook (Raz and Koornneef, 2001). Studies report that cell division occurring in the quiescent center induced by ethylene leads to the formation of additional layers of columella in the root cap, (Ortega-Martinez et al., 2007). Ethylene controls the root apical meristem size by signaling through the main canonical pathway and it also partially signals via two-component signaling via histidine kinase by regulating ARR1 (Street et al., 2015). Cell division rate is also controlled by ethylene at the time of differentiation of vascular tissue (Etchells et al., 2012).

1.11.2 Cell Elongation

Cell elongation in roots is inhibited by ethylene in dark-grown and green seedlings (Dolan and Roberts, 1995). It also promotes cell elongation in the hypocotyl of light-grown seedlings (Smalle and Straeten, 2006). Elongation of the petiole (F Millenaar et al., 2005) and elongation of root hair cells is also regulated by ethylene (Pitts et al.,

1998). Elongation of leaves and stems is caused by ethylene in plants grown underwater as a result of submergence stress (Jackson, 2007).

1.11.3 Cell Death

Programmed cell death (PCD) occurs in organ senescence, abscission, and defense against a pathogen attack in plants (Reape et al., 2008). Xylogenesis is a process of PCD in plants (Bollhöner et al., 2012) ethylene plays its role in xylogenesis. Increased levels of ASC and ACO resulting in high ethylene production are observed in Xylogenesis (Sorce et al., 2013) Ethylene induces aerenchyma formation due to submergence stress in water-logged plants under flood conditions to avoid the deficiency of oxygen is also an example of PCD in plants (Drew et al., 2000, Mühlenbock et al., 2007).

1.11.4 Seed Germination

Increased synthesis of ethylene takes place during seed germination (Corbineau et al., 2014). Ethylene not only recruits the germination of dormant seeds but also forces nondormant seeds to germinate under unfavorable conditions, (Corbineau et al., 2014). *etr1-1* and *ein2*-1 showed delay in germination of seeds increasing the dormancy period in seeds whereas *eto1* (ethylene over-producer) or *ctr1-2* promoted early germination of seeds (Corbineau et al., 2014).

1.11.5 Apical Hook Formation

Delicate hypocotyls of dicotyledonous seedlings form apical hooks when emerging from the soil after germination, this phenomenon protects the hypocotyl from any damage (Guzman and Ecker, 1990). Hookless1 (HLS1) an ethylene response gene forms an apical hook in seedlings (Lehman et al., 1996), this gene is triggered by ethylene response. Etiolated seedlings form exaggerated apical hooks in response to ethylene (Guzman and Ecker, 1990).

The apical hook is either absent or formed improperly in ethylene mutants that are insensitive and an exaggerated apical hook is formed in mutants that are hypersensitive to ethylene. *ein2* inhibits the formation of an apical hook (Smet et al., 2014). The exaggerated apical hook is formed by ethylene mutants that are constitutive (Takahashi-Asami et al., 2016). Two types of curvatures of apical hook are seen in

EIN3 mutants. *ein3* with the loss of function forms small curvature apical hook while over-expression of EIN3 increases the apical hook bending (An et al., 2012).

1.11.6 Growth of Hypocotyl

Ethylene responds differently in the hypocotyl in etiolated and light-grown seedlings. Short hypocotyl is seen in etiolated seedlings in response to ethylene as a triple response phenotype. While in light-grown seedlings hypocotyl length is promoted in response to ethylene. Ethylene levels are influenced by light in the regulation of hypocotyl growth. *eto2*, *ctr1,* and overexpression of EIN3 exhibit long hypocotyl in light, and short hypocotyl is seen in *etr1, ein2*, and *ein3/eil1*(ethylene insensitive mutants) (Smalle and Straeten, 2006, Zhong et al., 2012).

1.11.7 Ethylene Regulates the Growth of Primary Root

Ethylene regulates primary root growth. It inhibits the elongation of cells in the root meristem. It stimulates the division of cells of root apical meristem in the quiescent center (Ortega-Martinez et al., 2007). Small amounts of ethylene are needed to regulate the growth of roots (Tsuchisaka et al., 2009).

1.11.8 Ethylene Regulates the Development of Root Hair

Root hair growth is stimulated by ethylene (Tanimoto et al., 1995) *eto1* forms roots while insensitive mutants *etr1* & *ein2* form short root hair The emergence of root hair is controlled by ethylene. Root hair emerges from the apical end in ethylene producer (*eto1*) whereas in an insensitive mutant of ethylene (*etr1*), root hair emerges from the basal termination (Masucci and Schiefelbein, 1996). Ethylene controls the emergence site of hair in cells indirectly via auxin (Ikeda et al., 2009). Abnormal positions of root hair are observed in *ctr1* and ACC-treated wild-type seedlings. Ethylene regulates the differentiation of cells in the epidermal layer of roots (Tanimoto et al., 1995).

1.12 Biosynthetic Chemicals for Ethylene Experiments

ACC (1-aminocyclopropane 1-carboxylic acid) is used to synthesize ethylene in experiments. Etiolated seedlings show triple response in experiments while grown in ACC. 1-MCP (1-Methylcyclopropene) is ethylene's competitive inhibitor and antagonist. 1-MCP acts by binding to the ethylene receptors with high affinity as compared to ethylene binding to its receptor and therefore inhibits the ethylene binding

(Hall et al., 2000, Sisler and Serek, 2006). MCP competes to bind to receptors with ethylene (Blankenship and Dole, 2003). The affinity of binding to the receptor is greater in MCP in comparison to ethylene preventing the signaling of ethylene (Huber, 2008). 1-MCP delays the ripening and senescence in plants (Huber, 2008). AVG (Aminoethoxyvinylglycine) is used for inhibiting ethylene synthesis, it acts by blocking the action of ACS (1-aminocyclopropane 1-carboxylic acid synthase) that convert methionine to 1-aminocyclopropane 1-carboxylic acid in ethylene biosynthetic pathway. AVG is used in the experiments to control the endogenous levels of ethylene interfering with exogenous ethylene treatments (Beltrano et al., 1999).

1.13 Auxin as Plant Hormone

Plant growth hormone auxin controls the growth of the plant in conditions like light exposure and gravity as it can stimulate the growth towards light and gravity. (IAA) Indole-3-acetic acid occurs naturally in plants as auxin, it was identified by using in vitro bioassay in which the growth of oat coleoptile was stimulated when grown in auxin-containing agar blocks (J Bonner and Bandurski, 1952). Indole-3-acetic acid is broadly studied as a naturally existing plant hormone, which belongs to the family of amino acid tryptophan as they are chemically related. Tryptophan can be used to synthesize IAA in intact cells but this is not the only source as other synthetic pathways are also available. Auxin synthesis and its disassembly are done very precisely by plants, as it is very effective even in very low concentrations. Auxin is known to be synthesized in shoots and it moves from shoots to roots to perform its function. The cellular movement of auxin in plants is not well studied but it is known that the polar movement of auxin needs calcium ions (Ca^{2}) and membranes containing carriers are involved in its movement. Auxin plays in several plant growth processes such as the enlargement of the cell, plays a significant role in tropism, inhibits abscission, stimulates fruit development, and apical dominance of root and shoot. There are many auxins synthetically made such as naphthalene acetic acid, which are used to promote rooting in plants. Other synthetically produced auxins are 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) which are commonly used in killing weeds (Raven, 2013).

1.14 Evolutionary History of Auxin Biosynthesis in Plants

Agrobacterium is a plant pathogen, which produces auxins to capture plant cells for nutrient production. Plant pathogens such as *Agrobacterium* and *Pseudomonas* make use of tryptophan-2 monooxygenase, which is known as iaaM in the conversion of tryptophan to indole-3-acetamide, afterward hydrolase called iaaH, hydrolyzes it into Indole-3-acetic acid (Camilleri and Jouanin, 1991). Till now, the IAAM - IAAH pathway is one that only identified pathways depending on trp. But it is mostly thought that this iaaM / iaaH pathway is not used by plants to make IAA (Indole acetic acid). However, plant extracts have been identified to contain IAM and have been proposed as a key intermediate that is used to convert Indole-3-acetaldoxime (IAOx) to Indole acetic acid (Sugawara et al., 2009).

Another pathway is also found in some microbes through which tryptophan could be converted to IAA that is indole-3-pyruvate pathway (IPA). But this pathway unlike the iaaM / iaaH pathway, is not fully understood and studied in microbes. *Enterobacter cloacae* and *Azospirillum brasilense* has been used to clone the IPA decarboxylase, which is used in the reaction to convert the IPA into Indole-3-acetaldehyde. The genes that are responsible to convert tryptophan into IPA and enzymes that are responsible for reactions converting indole-3-acetaldehyde into the IAA have not been explored in microbes. It is still to investigate if the IPA decarboxylase present in microbes is useful in plants. Isolation of enzymes playing in the conversion of Trp to IPA is successfully achieved in *Arabidopsis* (Koga et al., 1992). Auxin in plants can be synthesized by both tryptophan-dependent pathways and tryptophan-independent pathways (Zhao, 2010). There are three tryptophan-dependent pathways found in plants for auxin biosynthesis. These are indole-3-acetamide, indole-3-pyruvic acid and indole-3-acetaldoxime. The significant auxin biosynthesis pathway in plants is indole-3-pyruvic acid (Qin and Huang, 2018). Auxin can be stored in the form of inactive conjugates such as oxIAA, intermediates formed in the degradation pathways (Garay‐Arroyo et al., 2012).

1.15 Auxin Transport

Auxin moves in plants through two types of mechanisms, one is the long distance that depends on the phloem and the other is the short distance that depends on the plasma membrane. Long distance carries auxin from aerial SAM to roots through phloem and

short distance depends on movements through plasma membrane import/export systems such as transporter or receptor-mediated, diffusion and secretion (Paponov et al., 2005). Plasma membrane carries influx carriers AUX1 (AUXIN RESISTANCE 1), LAX (LIKE AUXIN), and efflux carriers that are PIN (PIN FORMED) and ABCB (ATP BINDING CASSETTE GROUP B) used to transport auxin known as polar auxin transport PAT (Geisler, 2021). PAT controls the direction of auxin from one cell to another. Auxin distribution varies in different organs and parts of the plants and reflects the cellular activity in those parts (Vieten et al., 2007).

1.16 Significance of Auxins in Plants

Major functions of auxin are differentiation of vascular tissue, controlling elongation of the cell, inhibition of abscission, control of apical dominance, and several tropisms, and stimulation for the discharge of ethylene, which plays its role to increase fruit development (Peer et al., 2011).

1.17 Gene Regulation at Root Apical Meristem

During embryogenesis, the first event in the development of the root is the organization of an apical and basal axis (Zhang et al., 2013). At the quiescent center, the maintenance of an auxin maxima plays an important role with minimum mitotic activity. A Family of genes known as PLETHORA(PLT) is responsible for stem cell specification in the stem cell niche of root apical meristem in roots (Santuari et al., 2016). PLETHORA levels are controlled by different gradients of auxins, although other factors may also be involved (Galinha et al., 2007, Matsuzaki et al., 2010). Plethora genes are expressed in a graded manner in different zones of the root. High expression is found in stem cells and low expression is found in cell proliferation and differentiation zones (Bitonti and Chiappetta, 2010). Auxin also controls the regulation of genes known as WOX5. Expression of WOX5 occurs in the QC. WOX5 (WUSCHEL RELATED HOMEOBOX 5) genes are very important for maintaining the undifferentiated state of stem cells of the columella (Garay-Arroyo et al., 2012).

1.18 Root Hair Elongation

The surface area of roots is increased by the formation of root hairs that allow improved uptake of water and nutrients. The structure of each hair has a thin cylindrical structure that is formed from epidermal cells. Root hair is formed from epidermal cells following two major steps. The first step is fate determination which

decides if the cell produces hair or if it is a non-hair cell. The second step involves morphogenesis, which involves the root hair initiation and its elongation from the epidermal cell. A well-characterized gene regulatory network decides the formation of root hair in the epidermal cell (Grebe, 2012). Auxin greatly influences the morphogenetic step of root hair. Auxin takes part in the positive regulation of elongation of root hair (Yi et al., 2010).

1.19 Auxin Signaling

Auxin signaling starts following the perception of auxin by its nuclear receptor complex known as TIR1, which is the Transport Inhibitor Response1 receptor of auxin. TIR1 receptors are the F box subunits of E3 ubiquitin ligase complex SCF. Upon binding to auxin, TIR1 targets Aux/IAA for degradation. Aux/IAA are the repressors of the transcriptional response of auxin-responsive genes. In auxin's absence, AUX/IAA repressors are bound to ARF (Auxin response factor) on ARE and inhibit auxin response gene expression. (Kepinski and Leyser, 2005). Upon Auxin-TIR1 binding AUX/IAA is targeted for degradation. This proteasomal degradation releases the ARFs which then activate the transcriptional response of auxin. (Benjamins and Scheres, 2008).

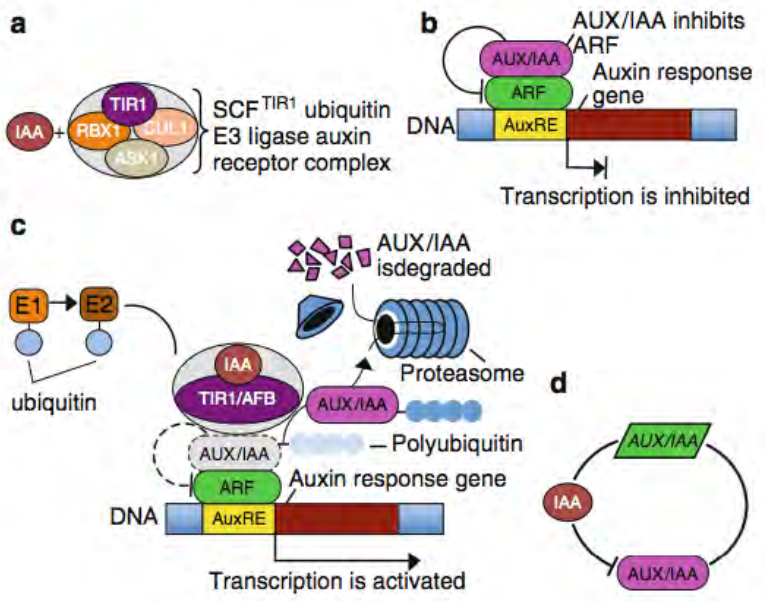

Figure 1.3: Schematic Illustration of Auxin Signaling (a) IAA binds to the receptor TIR, E3 ubiquitin ligase complex. (b) Auxin repressors AUX/IAA are bound to ARF

and stop ARF to cause the auxin response. (c) IAA binds to the receptor TIR which is complex with E3 ubiquitinates AUX/IAA for degradation and releases ARF to transcribe the auxin response (Taiz and Zeiger., 2003).

1.20 Cytokinins

Cytokinin was identified in the 1940s -1950s, when various elements present in different substances starting from yeast extract to tomato juice were studied. It was revealed that they could recruit and maintain plant stem tissues in culture along with auxin. Strong stimulation of cell division was seen in coconut milk, showing the greatest positive effect, signifying that it comprises a material that can incite cell division (Caplin and Steward, 1948). Sperm DNA was also found to be a strong activator of cell proliferation by Folk Skoog and Carol Miller in the 1950s when they cultured pith cells of tobacco with autoclaved herring sperm DNA (Miller et al., 1956). In sperm DNA they found that the derivative of adenine, 6-furfurylaminopurine, is the compound that is active and stimulates proliferation and named it kinetin. Stimulation of proliferation by tobacco pith cells in culture was done by kinetin in the presence of auxin. Zeatin present in immature maize endosperm was later identified as a naturally occurring cytokinin and was found as the biggest cytokinin present in coconut milk (Parker and Letham, 1973). After the discovery of cytokinin, they were used in many plant growth and developmental processes such as cell proliferation, initiation of the shoot and its growth, leaf senescence, apical dominance of root and shoot, uptake of nutrients, the relationship between sink and source, phyllotaxis, vasculature, formation of gametophyte, and many photomorphogenic developmental processes (Kieber and Schaller, 2014b). There are many natural cytokinins, which are adenine derivatives with different replacements present at the N^6 position of the adenine ring. One of the most common and abundantly present cytokinins is trans-zeatin found in *A. thaliana*, the isoprenoid side chain is found in the cytokinin most commonly (Gajdosova et al., 2011).

1.21 Cytokinin Biosynthesis

Cytokinins are derived from adenines. Different cytokinins are present with different N^6 substitutions present on the adenine ring. The cytokinin known as trans zeatin is an abundantly occurring natural cytokinin in plants with the isoprenoid side chain attached to the N^6 of the adenine ring. There are many other types of cytokinins such

as kinetin with a 6-furfurylaminopurine, 6-benzylaminopurine (BA) naturally occurring aromatic cytokinin. The synthesis of cytokinin from adenine is an enzymatic process. Conversion of AMP and dimethylallyl pyrophosphate into iPMP (N^6-(δ^2-isopentenyl) adenosine-5'-monophosphate) which is the active cytokinin was done by the enzyme which was recognized in *Dictyostelium discoideum* specie (Taya et al., 1978). *Agrobacterium tumefaciens* contains the *ipt* (isopentenyl transferase) gene that encodes the enzyme performing the same activity to activate the conversion of cytokinin (Akiyoshi et al., 1984). Many bacteria contain *ipt* genes and there are many crude extracts of many different plant tissues in which the activity of IPT was detected but unfortunately, it was not purified (Akiyoshi et al., 1984).

There are many different ways to synthesize free cytokinins. However, different enzymes are used in the synthesis of cytokinins. The first enzyme involved in the process is the isopentenyl pyrophosphate transferase (IPT). It uses ATP/ADP and DAMPP as substrates. iPRTP (isopentenyl adenosine-5'-triphosphate) and iPRDP (isopentenyl adenosine-5'-diphosphate), are the primary products, which are later converted into trans zeatin. IPT derives an isoprenoid side chain from DAMPP obtained from MEP (methylerythritol phosphate pathway) to form these ribotides (iPRTP and iPRDP). These ribotides (iPRTP and iPRDP) are then converted to trans zeatin ribotides by the P450 cytochrome enzyme by the hydroxylation of the isoprenoid side chain. (Takei et al., 2004). These trans zeatin ribotides are converted directly to their free base forms in one step via LONELY GUY abbreviated as LOG to active trans zeatin free base form, which is the active cytokinin found in plants. (Kurakawa et al., 2007). Active LOG enzymes are *encoded* by seven genes in *Arabidopsis* (Kuroha et al., 2009). Disorder of several *LOG* genes causes prominent growth retardations in root/shoot and defect in apical meristem maintenance, LOG7 and LOG4 play very important parts in the shoot apical Meristem & LOG3 and LOG4 play important roles in the root maintenance (Tokunaga et al., 2012). Expression of LOG4 is responsible for the formation of the L1 layer in SAM and also controls *Arabidopsis* floral meristem, and LOG4 is also assumed to play its role in the cytokinin synthesis in SAM derived apically (Chickarmane et al., 2012).

1.22 Cytokinin Metabolism

A decrease in the levels of cytokinin can occur by the conjugation of cytokinins to the glucose moieties and irreversible cleavage by cytokinin oxidases present in a cell causes a decrease in the levels of cytokinin. N^7 and N^9 are the nitrogen positions present in the purine ring of cytokinin that leads to irreversible N-glycosylation. Oxygen present on the zeatin side chains leads to O-glycosylation (Brzobohaty et al., 1993). O-glycosylation of cytokinin is considered a stable form for the storage of cytokinin. Because O-glycosylation can easily be converted by the B-glucosidases into the active form of the hormone. Conjugation of glucose to cytokinin makes it inactive when used in bioassays, they also cannot bind with the cytokinin receptors AHK (Spichal et al., 2004).

Cytokinin oxidases are present in many plant tissues that cleave the cytokinin N^6 side chains from cytokinin subsets present in the tissue. Cytokinin oxidase cleaves the unsaturated N^6 side chain of tZ and iP whereas the side chains of dihydrozeatin and BA (Benzyle adenine) are not cleaved by cytokinin oxidase. Preferred substrates for the enzyme are the free bases and their ribosides. O-glycosylation and N-glycosylation, of cytokinin also prevent cleavage by cytokinin oxidases (Kieber and Schaller, 2014b). The activity of cytokinin oxidase is inhibited by many synthetic cytokinins such as Thidiazuron and other urea-based cytokinins, the activity of cytokinin oxidase can be increased by the treatment of exogenous auxins (Bilyeu et al., 2001). Cytokinins can be inactivated by cytokinin oxidase therefore they can be used in the regulation of limiting effects of cytokinin. Cloning and characterization of cytokinin oxidase were first done in Zea mays kernel (Bilyeu et al., 2001). A peptide sequence of cytokinin oxidase was obtained and degenerate PCR (Polymerase chain reaction) was used to clone a gene. Previously it was believed that the Cytokinin oxidases are copper-dependent amine oxidases, but later it was found that it is a flavoprotein from the recombinant cytokinin oxidase from maize. These enzymes are amine oxidases that depend on FAD (Flavin adenine dinucleotide). There are seven known cytokinin oxidase genes encoded by the *Arabidopsis* genome and their members demonstrate distinctive expression patterns, properties of enzymes, and intracellular locations (Gao et al., 2014). Cytokinin treatment induces several CKX (Cytokinin oxidase) gene expressions. Endogenous levels of cytokinins can be reduced

by using the transgenic *Arabidopsis* lines overexpressing several CKX genes which can lead to various developmental defects (Bhargava et al., 2013).

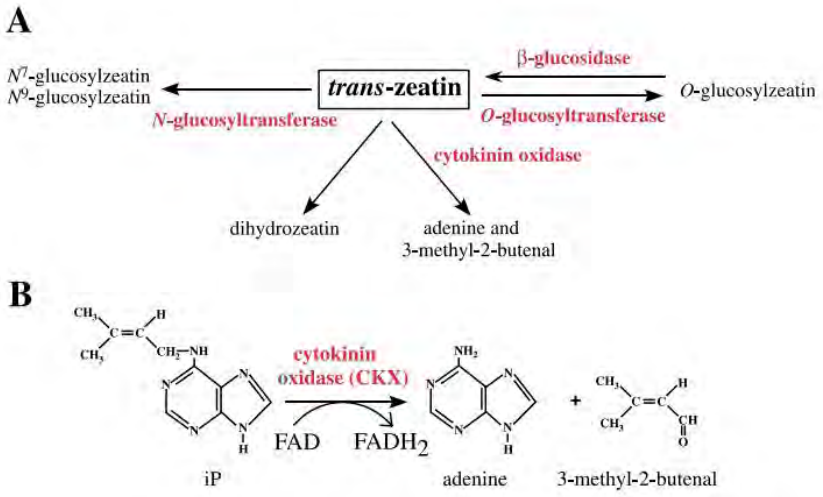

Figure 1.4: **Breakdown of Cytokinins.** (A) Trans-zeatin fate is shown. Cytokinin oxidase enzymes catalyze the reaction (Kieber and Schaller, 2014).

1.23 Cytokinin Transport

Previously it was believed that the synthesis of cytokinin occurs in roots only, but now in recent studies, it is revealed that cytokinins are synthesized in all the parts of the plant, with aerial tissues included (Kudo et al., 2010). A family of genes that are responsible for the cytokinin biosynthetic enzymes such as LOG and IPT are expressed in various locations in plant *Arabidopsis* that includes the aerial tissue as well. Cytokinin is transported in long distances mainly as tZ-riboside (tZR) in the xylem and the phloem is mainly transported as iP (Kudo et al., 2010). It is proposed based on the vascular localization, in plants tZR found in the xylem is used for transport towards shoots, and iP found in the phloem is used for transport towards roots (Kudo et al., 2010).

1.24 Plant Responses to Cytokinin

Cytokinins play an essential role in all the developmental phases of plants from cell proliferation and enlargement to the formation of flowers and fruits. They increase resistance to aging and adverse environment (Sakakibara, 2006).

1.25 Perception of Cytokinin and Its Signal Transduction

The cytokinin signaling occurs through a phosphorelay, which is similar to bacterial two-component systems. Bacteria use the two-component signal transduction pathway to sense the environmental stimulus and respond to it (Brenner et al., 2012). The two components system comprises a sensor kinase that is localized in the membrane through which it senses the environmental stimuli and passes it to a response regulator that transmits the signal, leading to the direct regulation of transcription of target genes. The signal is perceived by the sensor kinase through its input domain that autophosphorylates the histidine kinase domain. Histidine kinases are present in the form of dimers that transphosphorylate the conserved residues of histidine in the transmitter domain. Phosphorelay continues by transferring the phosphate to the receiver domain of the response regulator in the conserved aspartate residue. Response regulators consisting of a receiver domain also contain a DNA binding output domain and function as transcription factors (TFs) (Stock et al., 2000). *Arabidopsis* genome consists of protein-coding genes that are the same as the two-component system present in bacteria (Mizuno and Nakamichi, 2005, Schaller et al., 2008). These protein-encoding genes are present in the form of families such as histidine kinases, histidine phosphotransfer proteins (AHPs), and response regulators (ARRs) as transcription factors. Their role in phosphorelay signal transduction is studied in the genetic and biochemical analysis. Genetic analysis revealed that genes of a two-component system function in the cytokinin signal pathway; all the elements that play their role in cytokinin signaling were found to be present in early land plants like mosses (Pils and Heyl, 2009).

1.26 Cytokinin Receptors

Cytokinin receptors present in *Arabidopsis* are a family of histidine kinases and are made of three histidine kinases: (Arabidopsis Histidine Kinase) AHK2, AHK3, and AHK4. All these receptors have the same kind of structure which consists of transmembrane domains, a cytosolic domain, and an extra cytosolic. The extra cytosolic domain receives the signal and transfers it to the cytosolic domain for signal output. Cytokinin is perceived by the CHASE (the cyclase/His kinase-associated sensory extracellular) domain present in the extra cytosolic region (Heyl et al., 2013). The cytosolic domain consists of a histidine kinase domain and a receiver domain; that consists of a conserved residue. The receptor also consists of a second receiver domain

which is inserted between the histidine kinase and C terminal receiver domains. The second receiver domain diverges from other C-terminal receiver domains in the receptor in the sense that it lacks the conserved residues present in the domains. Aspartate residues are replaced by the glutamate residues in AHK3 and AHK4 (Heyl et al., 2007).

1.27 Binding of Cytokinin to its Receptors

A range of derivatives of adenine that display a cytokinin-like activity in plants can bind the receptor and recruit a response (Mok and Mok, 2001, Sakakibara, 2006). Crystallization of the CHASE domain present in AHK4 displayed the physical interaction of cytokinin with its receptor (Hothorn et al., 2011). Interaction of cytokinin with the CHASE domain is almost the same for all forms of cytokinin, but the trans-zeatin contains additional hydrogen bonds as compared to cis-zeatin, which provides a difference in their affinity towards the receptor. The binding site consists of almost 20 amino acids and is small in size (Daudu et al., 2017). Cytokinin conjugates being too large cannot bind to the small binding site. The difference in the specificity of cytokinin receptors is physiologically important (Kudo et al., 2010).

1.28 Histidine Containing Phosphotransfer Proteins

Histidine-containing phosphotransfer proteins commonly known as AHPs in *Arabidopsis* function as a signaling molecule present below the histidine kinase receptors of the cytokinin signaling pathway. The phosphoryl group is transferred by AHPs from the receptor's receiver domain to the response regulator's receiver domain. Five HPt (Histidine phosphotransfer proteins) are encoded by the *Arabidopsis* genome that consists of conserved residues needed for its action as a site of phosphorylation and one pseudo-HPt that deficits the phosphorylation site (Degtjarik et al., 2013). All HPts consist of a phospho transmitter domain with conserved histidine residues for phosphorylation. AHPs are mobile elements used for transferring phosphate groups from cytokinin receptors located in ER membrane to the nuclear-localized response regulators (Schaller et al., 2008).

Analysis of Yeast two-hybrid revealed that AHPs are capable of interacting with both histidine kinases in the receptor and the response regulators (Suzuki et al., 1998). It was revealed that cytokinin signaling does not affect the subcellular circulation of the AHPs, the distribution of the AHPs remains constant in the cytosol as well as the

nucleus whether the cytokinin is present or absent (Punwani et al., 2010). There is stable and dynamic distribution of AHPs continuously moving back and forth from cytosol to the nucleus without depending on the cytokinin and their status of phosphorylation. Numerous purposes are served by the distribution of these AHPs from the nucleus to the cytosol and vice versa. Phosphorylation of AHPs recruits it to the nucleus and transmits the cytokinin signal to the response regulator (Suzuki et al., 2001).

1.29 Type-B ARR Functions as TFs

Type-B response regulators mediate the initial response at the transcriptional level in the cytokinin pathway. *Arabidopsis* genome encodes the total eleven type-B ARRs, which are then divided into three subfamilies according to phylogenetic analysis: Seven ARRs are present in subfamily 1 (ARR1, 2, 10, 11, 12, 14and 18) and two members are present in the subfamily 2 (ARR13, and 21) and third subfamily consists of two (ARR19 and 20) (Kieber and Schaller, 2014, Mason et al., 2005). Type-B ARRs contain a receiver domain with a large C-terminal extension. The C-terminal extension consists of a conserved key feature i-e, a Myb-like DNA binding domain called as GARP domain because it was identified in GOLDEN2 maize (Hosoda et al., 2002, Imamura et al., 1999). Type-B response regulators act as transcription factors and are well explained by several evidences (Argyros et al., 2008). Expression of type B ARRs in yeast showed transcriptional activation which is carried out by type-B ARRs by binding to the target sequence of DNA directly by their GARP domain (Hosoda et al., 2002, Imamura et al., 2001, Sakai et al., 2000). The C-terminal region other than the GARP domain has variability, it also contains the nuclear localization regions and activation regions because of which the type-B ARRs are nuclear-localized. Interaction with other regulatory proteins is also facilitated by the C-terminal extension (Mason et al., 2005). Type B ARRs are regulated by protein degradation that functions as an additional mechanism in controlling their function. This makes the cytokinin pathway the same as the pathway of other hormones as their transcription factors are also targeted by the ubiquitin proteasomal degradation which includes auxin, ethylene, gibberellins, and jasmonate etc., (Santner and Estelle, 2010). But the stability of type-B ARRs is not much affected by the cytokinin as it is continuously degraded regardless of cytokinin's presence or absence. Different family members of Type-B ARR vary in terms of turnover (Kim et al., 2013, Kim et al., 2012) and this turnover depends on an

F-box, SKP1, Cullin, SCF E3 ubiquitin ligase complex. Four F-box proteins of KISS ME DEADLY determine the type B ARRS specificity towards the SCF complex. Yeast two-hybrid reveals the direct interaction of KMDs with different type B ARRs, ARR12 and ARR1 show the strongest type of interaction (Kim et al., 2013). These two types of type-B ARRs show a substantial contribution toward the *Arabidopsis* cytokinin signaling pathway (Argyros et al., 2008, Ishida et al., 2008).

1.30 Type A ARRs Act as Negative Regulators

Ten types of type A ARRs are encoded by the *Arabidopsis* genome and are divided into five pairs based on having high similarity in amino acid sequences (D'Agostino and Kieber, 1999, Schaller et al., 2008). Type A ARRs consists of a receiver domain that is different from type-B ARRs, in the sense that it lacks a classic domain to carry the output signal for the regulation of transcription. Type-A ARRs have very similar amino acid sequences present in the receiver domain, with more divergent sequences present in the short N- and C-terminal extensions which may function to produce specific downstream signal outputs (D'Agostino et al., 2000). According to the genetic analysis ARR3,4,5,6,7, 8, 9, and 15 act in negative regulation of cytokinin signaling, therefore reducing the sensitivity of cytokinin signaling by taking part in a negative feedback loop (To et al., 2007).

Type A ARR's negative regulation of cytokinin signaling can be controlled by two distinctive mechanisms. First, competition of type A ARRs with type B ARRs to get phosphorylated by AHPs as they are capable of interacting with it (Dortay et al., 2006), consequently the relative strength of two classes of response regulators will then cause a direct change in cytokinin pathway. An increase in the number of the phosphorylated type-A ARRs leads to a reduction in active type B ARRs as they are unable to get phosphorylated, and a resultant reduction in the transcriptional signal output (D'Agostino et al., 2000). Phosphorylated type-A ARRs take part in interacting with regulatory proteins, such interaction is also observed in bacteria consisting of a single-domain carrying response regulator (Jenal and Galperin, 2009).

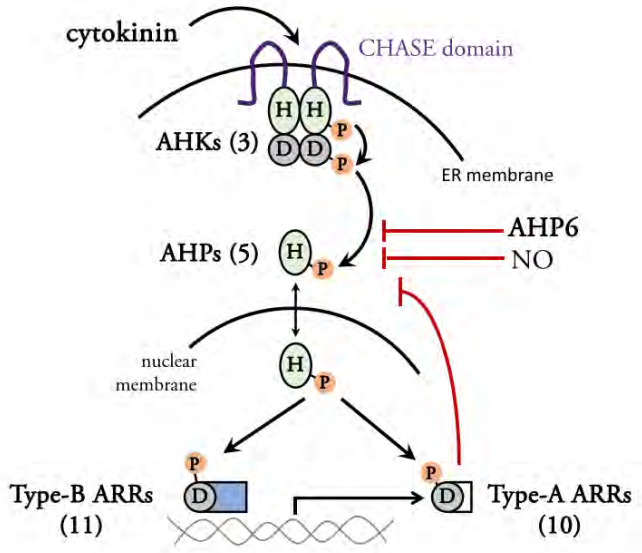

Figure 1.5: Potential Model of Cytokinin Phosphorelay Signaling Pathway. Cytokinin receptor AHK 2-3 with its CHASE domains is located within the lumen of the ER. The transmitter domain is activated by cytokinin binding and gets phosphorylated on His. Transfer of phosphate takes place on the Asp of the receiver domain. AHP protein carries this phosphate to the nucleus and transfers it to type B ARRs to carry out gene regulation. AHPs also phosphorylate type-A ARRs, which function in feedback that can hinder cytokinin signaling which is indicated as ^. AHP6 which is the pseudoHPt protein and nitric oxide (NO) functions in negative regulation of cytokinin transduction (Kieber and Schaller, 2014).

1.31 Role of Cytokinin in the Development of Root:

Cytokinin plays an essential role in the early development of roots. The development of the stem cell niches greatly depends on auxin, forming the auxin maxima at the stem cell niche and different gradient concentrations throughout the root development. But lack of ARR7 and ARR15 in cytokinin signaling has led to the formation of defective stem cell niches. This shows the involvement of cytokinin signaling in the formation of stem cell niches. Cytokinin does not play any significant role in the quiescent center and stem cell division. But cytokinin has a very significant role in determining the size of RAM. Cytokinin promotes the differentiation of cells in the meristematic zone and inhibits cell division (Růžička et al., 2009).

1.32 Hormonal Crosstalk in the Development of Root

The development of the primary root is the basic feature of the underground root system. Primary root development takes place embryonically after the germination of the seed. It plays a vital role in the development of plants by absorbing minerals, nutrients, and water from the soil. It also provides support and anchorage to the growing shoot of the plant. Plant roots help tolerance against adverse environmental and climatic effects. Plant roots change their growth patterns including the length of the primary root and the secondary root growth patterns (Saini et al., 2021). The growth and length of the primary root depend on two processes; the division of root cells and the elongation of root cells. These processes in the root are controlled by different hormones that act synergistically or antagonistically with each other (Qin et al., 2019).

Maintenance and changes in the cell division and elongation zone of the root in response to different environmental stimuli largely depend on SHY2 (SHORT HYPOCOTYL2) (repressor of auxin). It controls the size of the root meristem. Loss of function mutants of *shy2* in plants leads to the larger root meristem size. This large size is since in the absence of SHY2 differentiation of cells delay. Low levels of SHY2 lead to auxin signaling which promotes cell division at RAM. Cell division and elongation/differentiation balance is disturbed. Whereas high levels of SHY2 lead to shorter meristem (Pacifici et al., 2015). SHY2 acts at the transition zones of the vascular tissues and regulates the PIN expression and auxin distributions. Cytokinin signaling inhibits cell division and promotes cell elongation and differentiation. It recruits SHY2 via type-B ARR. Cytokinin inhibits the division at RAM and auxin promotes the division at RAM, both converge on SHY2 (Schaller et al., 2015).

Ethylene and Auxin interact with each other and work synergistically to inhibit the elongation of root cells. Ethylene is involved in the basipetal distribution of auxin in the root's elongation zone. Accumulation of auxin in the root tip in response to ethylene inhibits the elongation of root cells in root growth regulation (Růzicka et al., 2007).

1.33 Apical Meristems

Morphogenetic development of the embryo is usually defined by the establishment of apical and basal patterns along the longitudinal and radial axes during embryogenesis.

Shoot Apical Meristem is situated in between two cotyledons, spatially separated from the Root Apical Meristem (RAM) exhibited by the mature embryo along the longitudinal axis. The development of radial patterns relies on three tissues named protodermis, ground meristematic, and procambial region, these tissues give rise to cells that differentiate finally into endodermis, epidermis, cortex, and several other vascular tissues (Kropf et al., 1999).

1.34 Stem Cell Niche of Root Apical Meristems

During postembryonic development, all the root tissue cells are produced from the RAM which is a particular structure existing at the root tip of plants that gives rise to the mitotically active cells (Dinneny and Benfey, 2008). The undifferentiated cells present in the division and elongation zone of the RAM define root growth. RAM consists of stem cells that self-renew to generate daughter cells. These daughter cells divide in the division zone and differentiate into a specific cell forming the pattern of the root (Schaller et al., 2015). These stem cells are present in the root apex surrounding less mitotic cells present in a small group called the quiescent center (QC). In this restricted microenvironment, meristematic cells can exist for possibly an unspecified phase of time and give rise to progeny cells by their renewal, therefore revealing the key qualities of QC (Morrison and Spradling, 2008). QC together with self-renewing stem cells form the stem cell niche (SCN) of RAM (Schaller et al., 2015). These cells go through three different zones i.e., division, elongation, and differentiation. Regulation of the cell division and elongation/differentiation determine the rate of growth at RAM (Bennett and Scheres, 2010, Giehl and von Wirén, 2014, Petricka et al., 2012). RAM is maintained by the occurrence of two processes at equal rates and that is the generation of new cells equals the rate of cell differentiation (Pacifici et al., 2015). The equilibrium among the assembly of newly produced meristematic cells and their movement regarding the differentiation course assures the well-maintained condition of RAM (Shishkova et al., 2008).

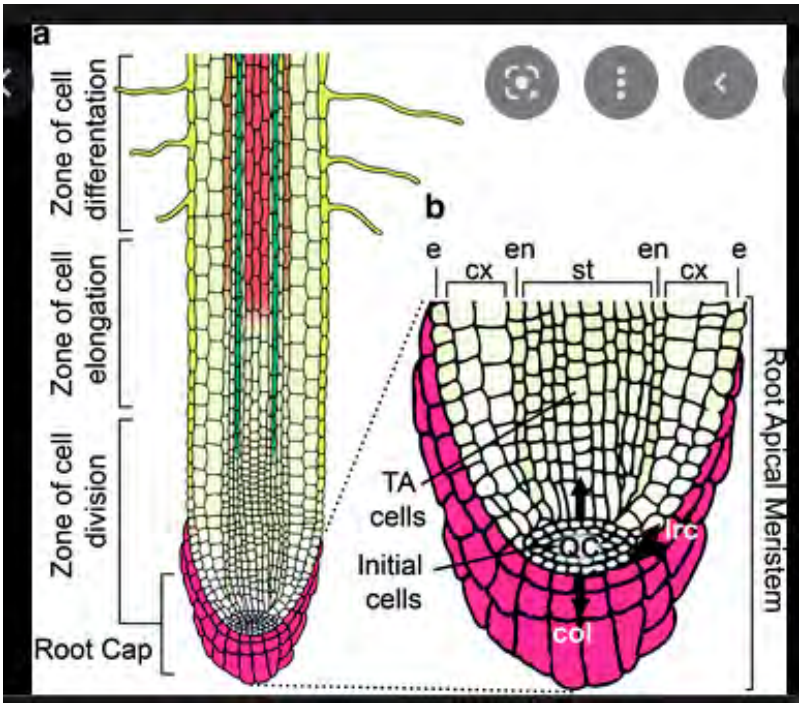

Figure 1.6: Structure of Root Apical Meristem (a). Longitudinal section of RAM showing zones of cell division, elongation, and differentiation. (b) The quiescent center with its surrounding cells forms a stem cell niche and differentiates into the stele, endodermis, cortex, and epidermis (Perilli et al., 2012).

1.35 Development of Root Apical Meristem

The organization of cells in the RAM of *Arabidopsis thaliana* seedlings was very well explained by (Dolan et al., 1993). The RAM of *Arabidopsis* consists of a dome characteristically structured as a closed meristem with three tiers, which consists of initials from distinctive layers of the cell to form a stele, next to the stele are endodermis-cortex, and root-cap-epidermis RCE tissues. The QC is comprised of four founder cells known as stem cell niches by the initials surrounding it. These are columella cells, stele cells, root cap-epidermis cells, and endodermis-cortex cells. According to the asymmetrical division, the RCE initials are primarily dividing periclinal to form one cell of lateral root cap and one remaining initial cell. The inner cell that preserves the initial's role then divides anticlinally making an epidermal cell and one initial cell (Perilli et al., 2012). Then, two uninterrupted consecutive asymmetric divisions of the EC initial cells give rise to the cortical and endodermal cells, EC initial cells primarily divide an anticlinal retaining one as initial with the quiescent center upholding the stem-cell fate, whereas the other derivative cell

undergoes periclinal division giving rise to an outer and inner cell, thus differentiating into the endodermis and the outer cortical layer. Ultimately, the periclinal division of the Stele initial cells gives rise to the xylem alongside the central axis and phloem tissues. Pericycle is formed by the division of outermost stele initials, which divide transversely. Central cells present in the root cap are formed by the periclinal division of columella initials distally that occur underneath the QC (Perilli et al., 2012, van den Berg et al., 1995). Root Apical Meristem is the main center where cell division occurs; it consists of a quiescent center (QC), and a population of mitotically active cells that are derived from stem cells present around the QC. These cells then transit from one zone to the other; meristematic cells after dividing ultimately stop dividing and transit into the elongation zone and then into the differentiation zone. The growth of the root is controlled by the different interacting phytohormones (de Vries et al., 2016).

1.36 Determination of RAM Size and Its Pattern Through Hormonal Circuitry

Plant development involves the distinct aspect of plant hormones that can act in a synergic way or antagonistic way in numerous developmental events. Modern improvements have been made to determine the interaction between different hormones to control the root growth pattern (Moubayidin et al., 2009). Meristem size of the root is maintained by a balance of the cell division rate of stem cells and movements of dividing cells in the meristematic zone to the elongation where the rate of cell elongation is maintained and leads to cell differentiation in the differentiation zone. Multiple hormones play their role to create this balancing cell division and cell differentiation. In these developmental phases, phytohormones act in two different ways either they act synergistically with each other or they act antagonistically. Regulation of cell division and differentiation in roots, by many different hormonal cross-talks, are well studied and acknowledged but there is a lack of studies reporting the organization and maintenance of Quiescent centers consisting of root stem cells (Lee et al., 2013). Auxin controls the growth of almost all the organs of the plants. It is synthesized mainly in the leaves and shoot apical meristem. From leaves, it is transported to roots through the phloem. It is also believed to be synthesized in the roots and maintains growth patterns of RAM with its different gradient concentrations (Garay‐Arroyo et al., 2012). Auxin is produced at both aerial SAM and underground RAM. In roots, auxin is distributed differently in division and elongation zones. In the

quiescent Centre, where very little cell division takes place auxin distribution is high. Stem cell fate is maintained by the auxin-inducible PLETHORA genes. High levels of PLETHORA keep the cells in an undifferentiated state and maintains the stem cell niche. Stem cells carry intermediate mitotic activity and have intermediate levels of auxin. The meristematic zone with high proliferation of cells has lower levels of auxin and the lowest levels of auxin in the meristematic zone lead to proliferation arrest and elongation (Garay‐Arroyo et al., 2012). Triple mutant *taa1, tar1,* and *tar2* produced short meristem in *Arabidopsis*. Quintuple mutant *yucQ* also produced short primary roots with very short meristem. Overexpression of any of the YUC genes in *Arabidopsis* also leads to root growth inhibition, which means that a basal level of auxin is required in meristem for the maintenance of root growth (Qin and Huang, 2018). Auxin in the meristematic zone promotes cell division by inhibiting the endoreduplication of cells. The distribution of auxin in roots is largely controlled by the PIN carriers in the polar transport system. The continuous acropetal flow of auxin towards the QC is mainly controlled by PIN1, PIN3, PIN4, and PIN7. PIN3 and PIN7 also control the lateral distribution of auxin towards the epidermis and lateral root cap (Mironova et al., 2010).

1.37 Auxin and Cytokinin Interaction

Cytokinins and auxin antagonistically interact with each other to control the patterning of roots throughout the postembryonic developmental process (Moubayidin et al., 2009, Muller and Sheen, 2008, Werner and Schmulling, 2009). Auxin and Cytokinin play an important role during embryogenesis, for the arrangement of the initial root stem cell niche, manifested by the maximum concentration of auxin in a single cell. At hypophysis high levels of auxin are responsible to activate the two negative regulators in cytokinin signaling, hence reducing the output of cytokinin (Muller and Sheen, 2008). After the embryonic stage of the root, cytokinin stimulates the differentiation of cells at the borderlines between the division and elongation zone called the transition zone. Cytokinins control this transition by the suppression of auxin signaling and its transport; similarly, auxin stimulates cell proliferation by cytokinin signal suppression (Blilou et al., 2005, Dello Ioio et al., 2007, Ruzicka et al., 2009). In particular, the meristem size of roots is reduced in increased cytokinin levels and impedes root growth, and this is achieved by the modulation of PIN expression and

thus the distribution of auxin. This crosstalk depends on the convergence of both hormones on one target gene, SHORTHYPOCOTYL (SHY2), IAA repressor protein is encoded by SHY2 which inhibits the auxin signal transduction (Benjamins and Scheres, 2008). Particularly, auxin response genes are inhibited by SHY2 by negative regulation of PIN 1, 3, and 7 and thus inhibiting auxin transfer at the transition zone (Dello Ioio et al., 2008). SHY2 is oppositely controlled by auxin and cytokinin; auxin recruits the degradation of SHY2 protein, more clearly and broadly (SCFTIR1) which leads to the stable expression of PIN and distribution of auxin. On the other hand, cytokinin stimulates the expression of SHY2 through a two-component phospho relay pathway. Receptor AHK3 recruits the activation of ARR1 that interact with the promoter of SHY2 at the transition zone of vascular (Su et al., 2011). Hence, auxin transport is restricted in the presence of cytokinin, which leads to the antagonism of auxin-dependent cell proliferation, and as a result cell elongation occurs (Dello Ioio et al., 2008).

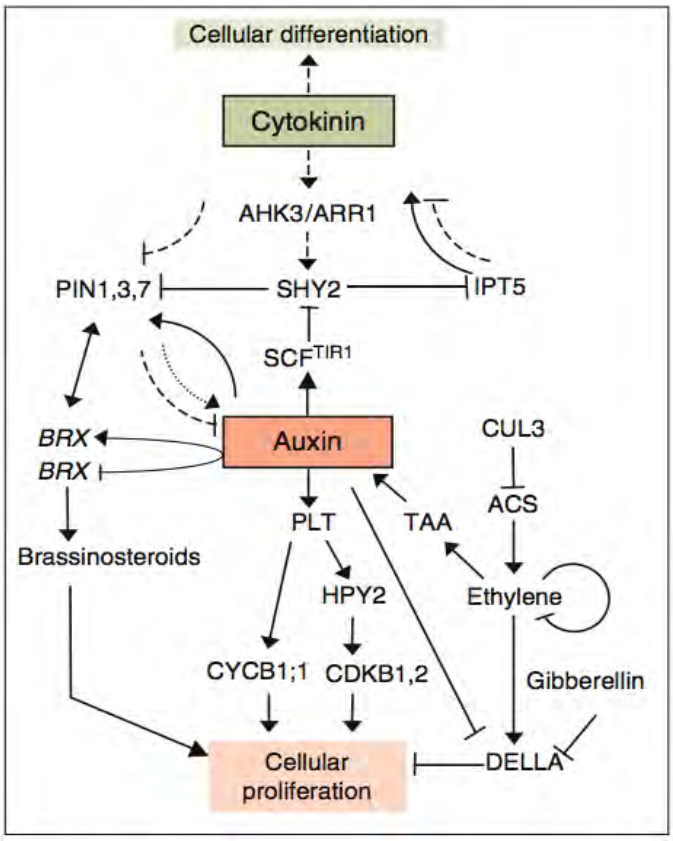

Figure 1.7: Schematic Illustration of Transcriptional Network Depicting Hormonal Interaction Lurking Root Patterns Cytokinin inhibits root cell proliferation by inhibiting auxin through PIN expression. Both hormones target SHY2, which is inhibited by auxin as it targets PIN expression when stimulated by cytokinins. Other hormones like ethylene are shown to promote DELLA to inhibit cell proliferation whereas gibberellin inhibits DELLA to help cell proliferation. Auxin promotes proliferation through cyclin-dependent genes and also through the activation of brassinosteroids (Stahl and Simon, 2009).

1.38 Interaction of Ethylene and Auxin

Root growth is controlled by the interaction of multiple phytohormones (García-Gómez et al., 2021). Auxin and cytokinin are well-studied in the root and are considered to be the principal players in the formation of the root. The whole process of root development starting from the embryonic root pole formation, stem cell niche and QC, control of mitotic activity in the meristem zone, and cell elongation and differentiation is largely controlled by auxin (Růžička et al., 2009). Regulation of root cell elongation by ethylene causes the inhibition of root growth of *Arabidopsis* without disturbing the activity of meristem (Liu et al., 2013). Root growth inhibition by

ethylene is constant with ethylene's old-defined function that it plays in cell elongation regulation (Hua and Meyerowitz, 1998). It is difficult to describe the ethylene and auxin interactions, knowing that ethylene inhibits the elongation of root cells, as we know that ethylene acts to stimulate the synthesis of auxin in the root along with directing its movement towards shoots, therefore increasing the activity of auxin in the zone of elongation where it inhibits elongation of the cell (Li et al., 2015). Cell proliferation can also be controlled by ethylene other than its well-categorized role to control cell expansion. According to genetic analysis, cell division occurring in the root apical meristem is regulated by CULLIN3 by using an ethylene-dependent process (Thomann et al., 2009).

The mechanisms that cause ethylene to negatively regulate the cell division at the RAM overlay with cytokinin action to carry out a similar function at RAM, and it may accelerate the cross-talk occurring between these pathways. Precisely, SHY2 and ARR1 are recognized as junctions between the ethylene and cytokinin pathway concerning their control of cell proliferation at RAM (Street et al., 2015b). Cytokinin controls the activity of auxin and its transport and causes an increased rate of cell differentiation by inhibiting division (Wu et al., 2021).

1.39 Ethylene Can Induce Cytokinin Through Its Histidine Kinase Domain

Cytokinin signaling takes place via a two-component system similar to the bacterial two-component system. Cytokinin's two-component system perceives cytokinin through a membrane-localized sensor kinase that autophosphorylates the histidine kinase domain. Ultimately, phosphorylation and activation of response regulators then propagate the signal. Cytokinin-induced genes are induced by a phosphorelay pathway (Schaller et al., 2011). *Arabidopsis* is used as a key model for the identification of key elements in the signaling pathway of ethylene. These identifications have led to the linear pathway of ethylene signaling that act through the perception of signal on receptors and activation of downstream CTR1, EIN2, and EIN3/EIL1 transcription factors for the propagation of the signal. A subset of ethylene receptors also consists of a histidine kinase domain and receiver domains similar to the two-component signaling system and seems to act through an alternative pathway other than the linear pathway (Shakeel et al., 2013). Ethylene receptors of subfamily 1 ETR1 and ERS1 contain a conserved histidine kinase domain, unlike subfamily 2 receptors. Subfamily 2 receptors contain a diverged histidine kinase domain that lacks the amino acid

residues required for enzymatic activity. Subfamily 1 receptors can therefore initiate a two-component phosphorelay signaling like cytokinin phosphorylating AHP and *Arabidopsis* response regulators ARRs (Street et al., 2015).

ETR1 receptor of ethylene propagates the signal upon receiving ethylene through canonical and noncanonical pathways. The canonical pathway operates similarly to all the receptors of ethylene through serine-threonine kinase CTR1, EIN2, and EIN3/EIL1. But the noncanonical pathway operates through a histidine kinase and propagates cytokinin signaling by activating AHPs and response regulators of the cytokinin signaling pathway. This crosstalk of ethylene and cytokinin regulates root apical meristem (Zdarska et al., 2019). Cytokinin treatment increased the biosynthesis of ethylene by increasing the levels of ASC5 in *Arabidopsis* etiolated seedlings (Chae et al., 2003). Expression of TCS: GFP, a sensitive cytokinin reporter was significantly increased by the ethylene. Ethylene increased the expression of TCS: GFP in the epidermal cells present in the transition zone of the root. Whereas cytokinin increased the TCS: GFP levels in the columella and central vasculature. The increased expression by ethylene was distinct from the expression observed by cytokinin (Zürcher et al., 2013). ETR1-mediated cytokinin signaling via its noncanonical interaction with AHPs was analyzed. Four AHPs were found to interact with ETR1 suggesting the interaction of ethylene with cytokinin. A mutation that inactivates the histidine kinase activity of ETR1 reversed the ethylene-induced cytokinin response in plants (Artner and Benkova, 2019). Ethylene and Cytokinin interaction control the root growth pattern in different developmental stages of roots. Exogenous application of cytokinin inhibits root elongation by inducing ethylene (Yamoune et al., 2023).

1.40 Objectives of the Study

According to a very well-known model, ethylene may inhibit the growth of roots in *Arabidopsis* by regulating root cell elongation without disturbing the activity of Meristem (Li et al., 2015). Maintenance of RAM activity is achieved by the opposite roles of cytokinin and auxin in which auxin stimulates the proliferation of cells at RAM and this auxin action is inhibited by cytokinin to reduce the size of RAM (Su et al., 2011) Interactions of the cytokinin and auxin pathways are well defined with other plant hormones, but till now, ethylene interaction with these hormones in controlling the activity of RAM has not been reported.

In this study, we aim to find that ethylene may also regulate the proliferation of cells together with its well-categorized function in the regulation of cell expansions. We, thus, decide to utilize pharmacological and genetic tools that will be very helpful for the analysis of the ethylene signaling pathway to depict its important role in regulating cell proliferation at the root apical meristem. We divided the objectives of this study into two parts for the ease of the reader, the objective of part A is that ethylene plays a significant role in controlling the RAM size through changes in cell proliferation and SHY2 acts as a convergence point for both cytokinin and ethylene in inhibiting RAM size. As ethylene uses the same point of convergence SHY2 as cytokinin, so there might be some kind of interaction between ethylene and cytokinin. The use of 1-MCP can inhibit the effect of ethylene to prove its role in inhibiting the RAM size.

We also aim to find the interaction between ethylene and cytokinin. We treated our plants of *Arabidopsis Thaliana* with ethylene and cytokinin together and individually to find the interaction between them.

In part B we also evaluated the role of 1-MCP in inhibiting ethylene in tomato fruit. Tomatoes have great agricultural importance. Developing countries like Pakistan face big problems in the storage and transport of tomatoes. These problems lead to the shortage of tomatoes in the country and the need to import from other countries. The role of 1-MCP in inhibiting ethylene delays the ripening process and increases the shelf life of tomatoes. We will check different physiological parameters and ripening-related genes in tomatoes treated with 1-MCP. The purpose of the objective is to increase the shelf life of tomatoes by inhibiting ethylene. And to check the changes in the expression pattern of ripening-related genes after 1-MCP treatment.

1.41 Objectives of the Study Part A

Part A: To find that ethylene inhibits the division of cells at root apical meristem and SHY2 is the point of convergence for ethylene in inhibiting root apical Meristem (RAM). To characterize the transcriptional cross-talks between ethylene and cytokinin signaling pathways.

2. Materials and Methods

To find the function of ethylene in reducing the size of root apical meristem by inhibiting the proliferation of cells we used different ethylene-insensitive and constitutive mutants. Cells present in the cortex layer of the RAM division zone were counted. Roots were treated with chloral hydrate to clear the tissue present in the roots and make the cells visible for the count. *Arabidopsis* seedlings both control and mutants were treated with 10uL/L ethylene with a syringe through rubber septum in a container and controls were kept in air for four days for etiolated seedlings and 9 days for green seedlings. Cells of the division zone in RAM were counted under the microscope. qRT PCR analysis of ethylene response genes and other genes expressed in the division zone were analyzed in response to ethylene. SHY2 gene that inhibits auxin in the division zone in response to cytokinin was analyzed against ethylene. 1-MCP was used to inhibit ethylene in root apical meristem. For the RAM experiment, 10uL/L 1-MCP was applied to *Arabidopsis* wild-type seedlings to inhibit the ethylene at RAM. The dose response of 1-MCP was checked in dark-grown seedlings to check its efficiency of inhibiting triple response in etiolated seedlings in ethylene's response. To find the interaction of ethylene and cytokinin we used wild-type and ethylene-insensitive mutants of *Arabidopsis thaliana*. We gave single treatments of ethylene and cytokinin to seedlings and we also gave concurrent treatment of ethylene and cytokinin and compared the results of single and concurrent treatment in wild type with ethylene insensitive mutants. We made two types of experimental setups one for etiolated seedlings and the other for green seedlings. The same treatments of ethylene and cytokinin were given to both experimental setups. Green seedlings were used to find if there is any change observed in the interaction of ethylene and cytokinin due to light. Gene expression of ethylene-induced genes and cytokinin-induced genes was checked in response to treatments.

2.1 Plant Material

For the analysis of root apical meristem wild type, ethylene insensitive and constitutive mutants of *Arabidopsis thaliana* were grown on nylon mesh on MS media. The ethylene-insensitive mutants with Columbia background used were *etr1-1*, *ein2-50*, *ein2-1* and *ein3-1/ eil1-1*, and the constitutive ethylene mutants used were *ctr1-2*

and triple mutant *etr2-3/ ein4-4 /ers2-3* in the Columbia background. Other ethylene mutants of *Arabidopsis* with WS background were *etr1-9*, *ers1-3*, and *etr1-9 ers1-3*. To study the interaction of cytokinin and ethylene wild type *Arabidopsis* and ethylene insensitive mutants of *Arabidopsis thaliana* were grown on MS media. The ethylene-insensitive mutants with Columbia background used in this study were *etr1-1* and *ein3-1 eil1-1*.

2.2 Seed Sterilization

Seeds of wild type, ethylene insensitive mutants, and ethylene constitutive mutants were sterilized before plating them on growth media. Seeds were first washed with 70% ethanol. 20% bleach solution with 1% Triton X-100 was used for washing seeds for 20 min after ethanol. Seeds were then washed with sterile deionized distilled water several times in a sterile hood for the removal of bleach from seeds.

2.3 Plant Growth Conditions of Etiolated Seedlings for 1-MCP Experiment

Wild-type seedlings of *Arabidopsis thaliana* were placed on 0.8% (w/v) agar plates with (Murashige and Skoog) MS basal medium for growth, pH 5.70 was adjusted with KOH, Gamborg's vitamins were included in the medium (Media, Sigma, St. Louis). The dose-response of 1-MCP was analyzed in the presence of 1 uL/L ethylene to check the recovery of ethylene's inhibitory response by 1-MCP. Seedlings were kept in closed containers with a rubber septum. 1uL/L ethylene and 0, 1, 5, 10, 100, and 1000 1-MCP in nL/L was applied through a rubber septum and control was kept in air (without ethylene and 1-MCP). All the containers except control and 1-MCP treatment only, were treated with 1uL/L ethylene before 1-MCP. All the containers with treatments were kept for four days in the dark. Root lengths were measured with the ImageJ software.

2.4 Growth Conditions of Green Seedlings for the Analysis of Root Apical Meristem Experiment

Wild type, ethylene insensitive mutants and constitutive mutants of *Arabidopsis* were grown on MS basal medium containing Gamborg's vitamins and MES buffer, 1% of sucrose, and 0.8% of phyto agar for molecular and physiological analysis (Argyros et al., 2008). To facilitate the measurements of roots and maintain good growth conditions square plates were used and placed in a vertically oriented position. Seeds

were surface sterilized with ethanol and 1% Triton X-100 followed by stratification for 3 d at 4˚C in the dark and then shifted to a 22˚C growth chamber. Seeds were placed on nylon mesh placed on square petri plates containing MS media. Continuous light was provided for the green seedling, white light by fluorescent bulbs producing 18,000 K (Aqua-Glo; Rolf C. Hagen Corp., Mansfield, MA). 10uL/L ethylene was introduced into the container through a rubber septum. For the 1-MCP experiment to study its inhibitory effects of ethylene in RAM 10uL/L 1-MCP was applied in the closed container through rubber a septum with 10uL/L ethylene. To study the dose response of ACC, ACC was incorporated into MS media.

2.5 Root Meristem Analysis

To find the impact of ethylene on RAM, measurement of RAM size was done by counting the cells of the cortex layer starting from the QC (quiescent center) to the first cell present in the elongation zone with the Nikon Eclipse 90i optical microscope containing Nomarski optics with 20X objective (Perilli and Sabatini, 2010). Chloral hydrate was used to clear the root tips of seedlings for the determination of root meristem size. Chloral hydrate was prepared in an 8:3:1 mixture with 8 ul chloral hydrate: 3ul distilled water: 1ul glycerol. Root tips were placed on the slides and a drop of chloral hydrate was added to the roots. Roots with a drop of chloral hydrate were covered with a cover slip and the number of cells in the cortex layer of the root were counted under the microscope. Meristematic cells of wild-type seedlings were also counted using propidium iodide staining.

2.6 Experimental Setup for Etiolated Seedlings Used in the Experiment for Cytokinin Ethylene Interaction

Wild-type *Arabidopsis* and insensitive mutants of ethylene, *etr1-1,* and *ein3 / eil1* of *Arabidopsis thaliana* were grown on Murashige and Skoog media 0.8% (w/v) agar pH was adjusted to 5.70 with KOH. Media contains Gamborg's vitamins and 5uM AVG. To find the interaction of cytokinin and ethylene in etiolated seedlings, seedlings were grown on a nylon sheet kept on media. A nylon sheet was used for the easy transfer of seedlings to new plates for treatment. Seeds were stratified for two days and after stratification seeds were kept in light in a growth chamber for 20 hours at 22° C. For etiolated growth seeds were grown in the dark for four days. After four days seedlings on the nylon sheets were shifted to the freshly prepared plates of MS media containing

5uM AVG, and DMSO for control samples, MS media containing 5 uM AVG and 1uM BA for cytokinin treatments. For ethylene treatment seedlings were shifted to MS media containing 5uM AVG and shifted to a closed container with a rubber septum, 1uL/L ethylene was injected into the container. For the co-treatment of cytokinin and ethylene, seedlings were shifted to MS media containing 5uM AVG and 1uM BA and then shifted to a closed container for 1 uL/L ethylene treatment given with a syringe through a rubber septum. 2 hours of treatment was given to all the seedlings.

2.7 Growth Conditions for Green Seedlings Used in the Experiment for Cytokinin Ethylene Interaction

Wild-type *Arabidopsis* and insensitive mutants of ethylene, *etr1-1,* and *ein3 / eil1* of *Arabidopsis thaliana* were grown on MS basal medium containing Gamborg's vitamins and MES buffer, 1% of sucrose, and 0.8% of phyto agar with 5uM AVG for molecular and physiological analysis (Argyros et al., 2008). To analyze the interaction of cytokinin and ethylene in roots and shoots separate, seeds were stratified for two days and kept in continuous light for 7 days. After 7 days of growth seedlings were shifted to liquid MS media containing DMSO for control, 1uM BA for cytokinin, 10uM ACC for ethylene treatments, and 1uM BA and 10uM ACC for co-treatment of cytokinin and BA. Seedlings in liquid media under treatment were kept on a shaker under constant light for 2 hours. After 2 hours samples were collected separately for roots and shoots. Roots were cut with a blade and separate samples of roots and shoots were immediately shifted to Eppendorf-containing beads kept in liquid nitrogen to flash freeze the samples for further analysis.

2.8 RNA Extraction Protocol and DNase I Treatment of Green Seedlings

RNA isolation from wild type, ethylene insensitive mutants, and ethylene constitutive mutants for qRT PCR analysis. Isolation of RNA was done in three replicates each (biological replicates and experimental replicates) with RNeasy Plant Kit (Qiagen). To determine the molecular analysis of genes, present in RAM, root tips of 1mm in size were cut with a blade and immediately kept in Eppendorf with a metallic bead and shifted to liquid nitrogen. For the interaction of cytokinin and ethylene roots and shoots were separated for green seedlings. A mixer mill was used to grind the sample in

Eppendorf with a bead. 0.1g of tissue was ground in liquid nitrogen until a fine powder was made and was mixed well. 500 ul of RB buffer was used for the lysis of ground tissue and debris was then removed by filtering in homogenizer mini columns. 2 ml tubes containing homogenizer mini columns were centrifuged at 14000g for 5 minutes. After removing the debris with homogenizer mini columns, an equal volume of 75% ethanol was added after transferring the lysate to new 1.5ml tubes. The sample was thoroughly mixed in 75% ethanol with a pipette and 700 ul of the sample was transferred to new 2ml tubes containing Hi band RNA mini column. The sample in a 2ml tube containing Hi band mini-column was centrifuged for 1 min at 12000g. Filtrate was discarded and the step was repeated until all the RNA in the sample was completely shifted to the column. The column was washed with RNA wash buffer 1 and 2 subsequently. Filtrate was discarded and the empty column was centrifuged at high speed for 2 min for the removal of any trace ethanol. RNA was then eluted by washing the column with DEPC water. For DNase treatment, Qiagen Kit was used for the removal of DNA if present in samples according to the company's instructions. DNase digestion reagents were added to the RNA and incubation was done at 37° C for 30 min. Samples were again incubated for 2 min at room temperature after adding DNase 1 inactivation reagent. The sample was centrifuged and transferred to new tubes. A nanodrop spectrophotometer (ND/-1000) was used to quantify RNA at 260 and 280 nm. The sample was also run on an agarose gel to estimate the integrity of RNA.

2.9 First Strand cDNA Synthesis

First-strand cDNA was synthesized with the Superscript III First Strand cDNA Synthesis kit of Invitrogen with oligo dT primers followed by RT-qPCR according to the instructions. We used 1ug total RNA for cDNA synthesis.

2.10 Quantitative Real-Time PCR

Primers for the analysis of genes involved in inhibiting proliferation were designed with IDT (integrated DNA technologies) (Table 2.1). Primers for the analysis of ethylene and cytokinin-induced genes were also designed with IDT (integrated DNA technologies) (Table 2.2). The annealing temperature was set at 55-60˚C for the primers used. Relative quantification of all the selected genes was done by a 7500 Real-Time PCR system (Applied Biosystems). Three technical replicates of each

selected gene were run in qRT PCR. A housekeeping gene *β tubulin* was used as the internal control. The difference between the target gene and *β tubulin* provided the fold change and relative expression of target genes. 1 ug RNA was used to make cDNA and it was diluted five times for further PCR analysis.

The reaction mixture for qRT PCR was made by adding 5ul iTaq Syber green supermix from Biorad, 1ul of primers (F+R), 1ul cDNA, and 3ul DD H_2o. 10ul of the total reaction mixture was used. 96 well plate was used to run samples in the PCR. Thermal cycling was set for 40 repeated cycles with initial denaturation set at 95°C for 10 min followed by repeated cycles with denaturation at 95°C for 15 sec, annealing temp was set at 60°C for 1min, and elongation temp was set at 72°C for 30 sec.

Table 2.1 Primers for the Analysis of Root Apical Meristem

Gene	Forward (5'---- 3')	Reverse (5'----- 3')
SHY2	AAACAGAGCTGAGGCTGGGATTAC	AACTGGTGGCCATCCAACAATCT
ICK1	GTATCGACGGGGTACGAAGA	CCTCCCGCTACAACAACAAT
ATPN	AGTGACTTAGCCGTGAACACCACA	ACGAGACCGATCAACTCCCAAACA
ETR2	AGAGAAACTCGGGTGCGATGT	TCACTGTCGTCGCCACAATC
B-TUB3	TGGTGGAGCCTTACAACGCTACTT	TTCACAGCAAGCTTACGGAGGTCA

Table 2.2 Primers for Ethylene and Cytokinin-induced Genes

Gene	Forward (5'---- 3')	Reverse (5'----- 3')
ARGOS	GTCATGGACGTCGGAAGAAACAAC	GGGAACCAATAGCAGCATAAACGG
ERS1	TTCAGTCTACAAGCGATCTTTGAAG	AGCGCGACAAACCGTTTACAGAGA
ERF1	TCTAATCGAGCAATCCACGCAACA	AACGTCCCGAGCCAAACCCTAATA
ETR2	AGAGAAACTCGGGTGCGATGT	TCACTGTCGTCGCCACAATC
B-TUB3	TGGTGGAGCCTTACAACGCTACTT	TTCACAGCAAGCTTACGGAGGTCA
ARR7	AGAGTGGAACTAGGGCTTTGC	CTCCTTCTTTGAGACATTCTTGT
ARR15	CTGCTTGTAAAGTGACGACTGT	AGTTCATATCCTGTTAGTCCCG
CKX5	CCCCATGAACAGGCACAAGT	GAGGATCTCCCGGTTCTGCC

2.11 Statistical Analysis

Statistical analysis was accomplished by doing One-way ANOVA (ANalysis Of VAriance) with post-hoc Tukey HSD (Honestly Significant Difference). Comparison of different treatments within genotypes was done by Bonferroni and Holm multiple Test Calculator. (* $p < 0.05$; ** $p < 0.01$), statistics were applied to experimental samples and the wild-type controls in each case

3. Results and Discussion

3.1 To Find the Evidence of Ethylene in Inhibiting Root Apical Meristem (RAM) Size and Interaction of Cytokinin and Ethylene.

Ethylene is responsible for multiple roles in growth and regulation throughout different parts of the plant like cell division, cell elongation, cell death, seed germination, etc., (Schaller, 2012). After seed germination, the formation of apical meristem takes place, which is controlled by different hormones to balance the division and differentiation phases of cells during plant growth to its final size. The Root Apical Meristem (RAM) of the plant is divided into three different zones i-e, cell division, cell elongation cell differentiation. RAM, consists of a quiescent center that produces the initial cells that divide in three different directions i.e., proximally, laterally, and distally, hence producing layers of root cells, root cortex cells, root endodermis, root stele, lateral /epidermal root cap and columella (Taiz and Zeiger, 2003). Different hormones interact either synergistically or antagonistically with each other to control the growth of the root (Moubayidin et al., 2009).

Ethylene is known to inhibit RAM activity, reducing the root size (Le et al., 2001). Auxin recruits cell division of the Root apical meristem and cytokinin antagonizes this activity and decreases the RAM size. Auxin and cytokinin crosstalk at RAM and their interaction with other plant hormones are well described, but the role of ethylene in controlling the RAM size is not well studied. We used different ethylene mutants and wild types of *Arabidopsis thaliana* to study the role of ethylene at RAM. We also used 1-MCP to study the signaling of ethylene to reassess its role in cell division with its main focus on the root meristem. As both cytokinin and ethylene inhibit RAM and ethylene has histidine kinase activity in its receptor like cytokinin. We assume that ethylene can induce cytokinin signaling and there is some kind of interaction between these hormones. To find the interaction of ethylene and cytokinin and if ethylene can induce cytokinin via its two-component system like histidine kinase, we performed various experiments at the transcript level. We have multiple treatments of ethylene and cytokinin to wild-type and ethylene mutants of *Arabidopsis* and compared the results of ethylene and cytokinin-induced genes in response to treatments. According to our predicted model (Fig.3.1) ethylene and cytokinin crosstalk via interaction of

their transcription factors EIN3 and ARRs. The interaction of their transcription factors might implicate a combined effect on the regulation of genes.

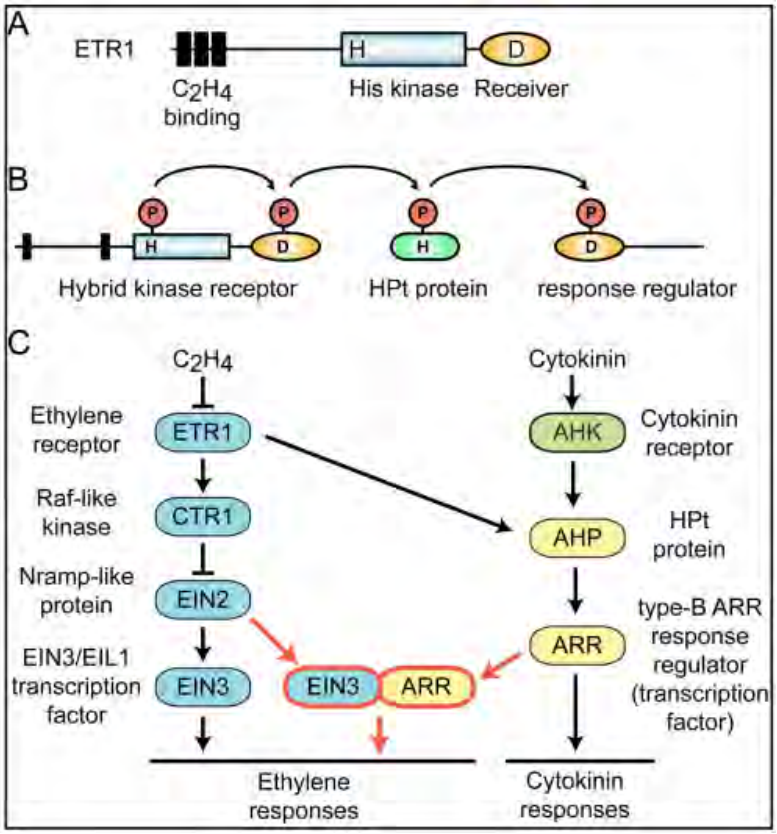

Figure 3.1: Model depicts the signaling via histidine kinase receptor. (A) ETR1 receptor of ethylene with its histidine kinase and a receiver domain. Transmembrane helices of receptors are shown in black rectangles. Histidine kinase is shown in a big blue rectangle with a yellow oval receiver domain. (B) Phosphorelay activating histidine phosphotransfer proteins (green capsule) and a response regulator (yellow oval) via histidine kinase. H and D alphabets indicate the conserved phosphorylation sites of aspartate and histidine. P indicates phosphorylation. (C) Components of ethylene signaling pathway and cytokinin signaling pathway. The black arrow indicates the possible interaction of ETR 1 of ethylene with histidine phosphotransfer proteins of cytokinin. Orange arrows indicate the interaction of ethylene and cytokinin via Ein3 and ARRs and their combined action on ethylene response genes.

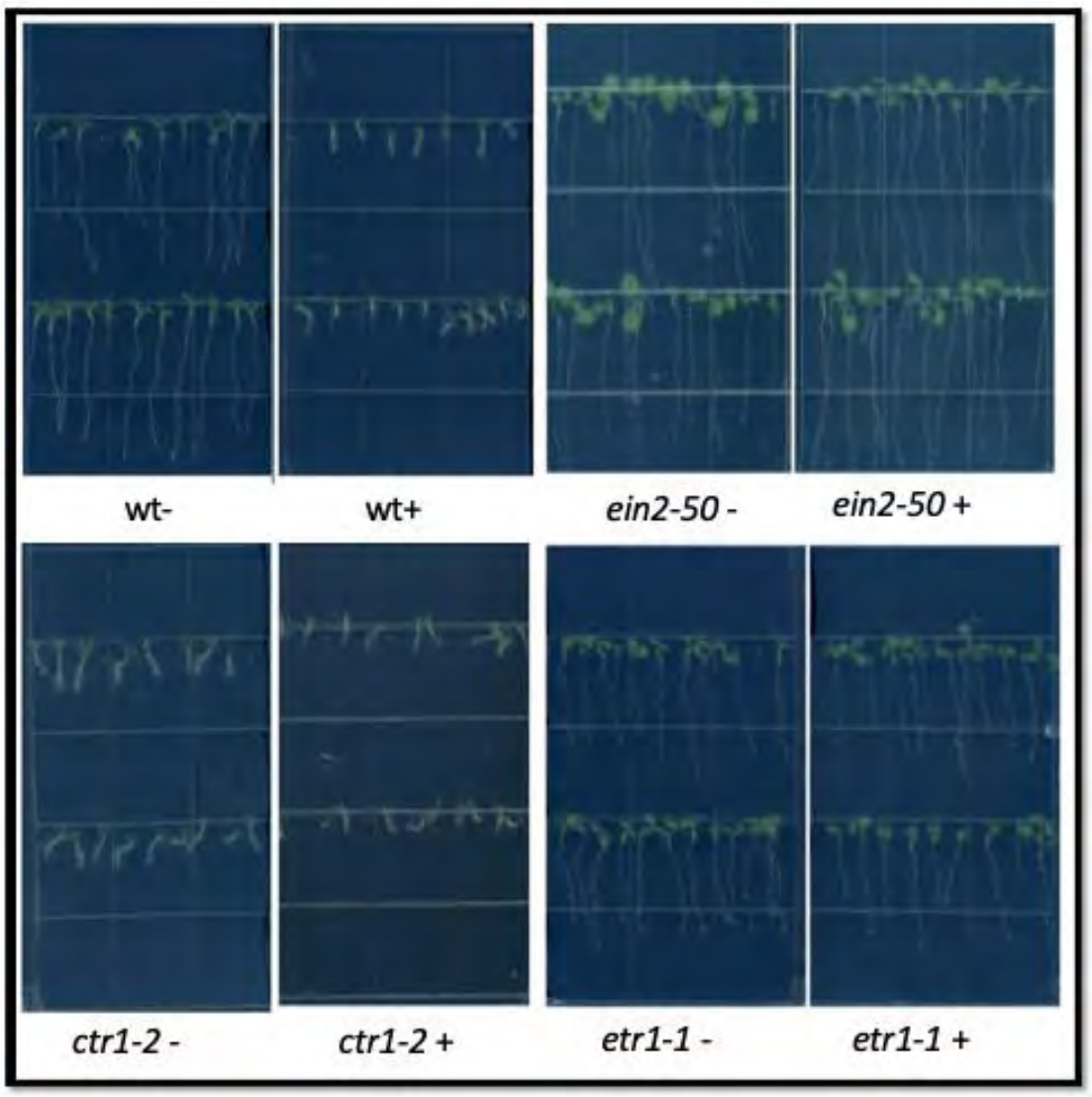

Figure 3.2: Wild Type and Ethylene Mutant Phenotypes. *Arabidopsis* wild type, *ctr1-2*, *ein2-50,* and *etr1-1* were used for the phenotypic study of ethylene response. Seedlings were kept in 10 µL/L ethylene under continuous light for growth for nine days. Control samples were kept in the air.

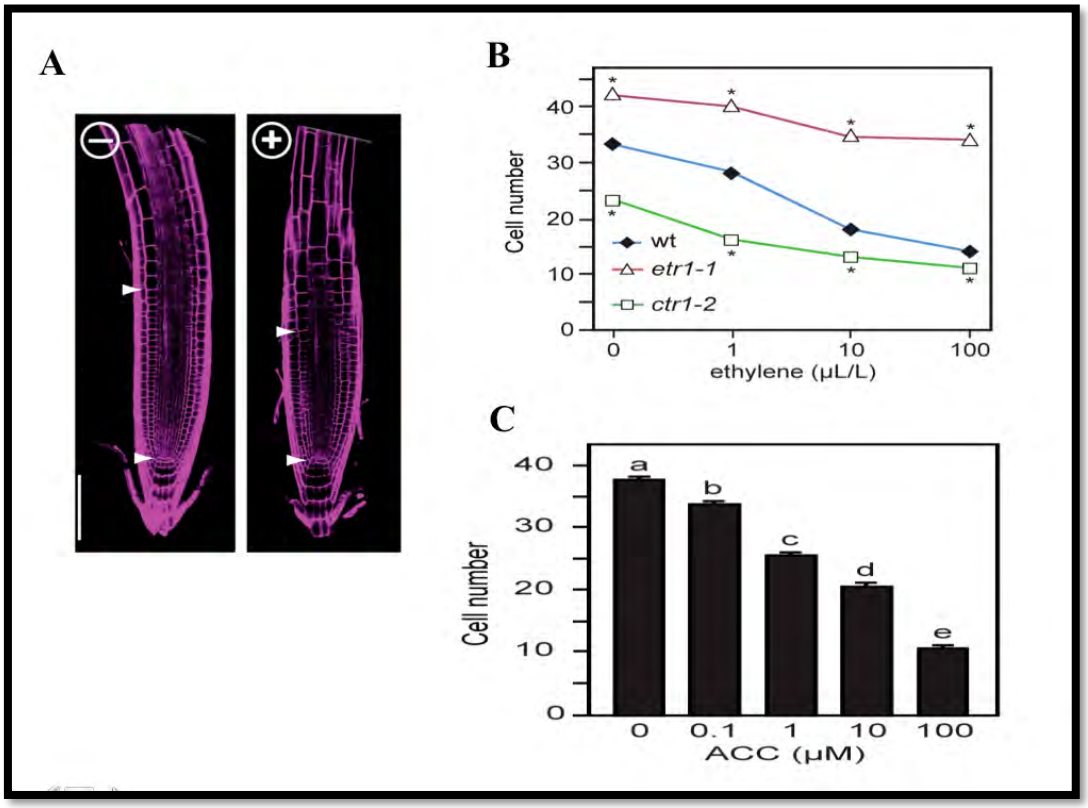

Figure 3.3: Negative Regulation of Ethylene at Root Apical Meristem. (A) Root Apical Meristem of *Arabidopsis* wild-type seedlings in (+) and (-) 10uL/L ethylene. Propidium iodide was used for fluorescent visualization. In the figure arrows below show the site of QC and the arrow above indicates the first elongated cell. **(B)** Cell number of RAM of wt, *ctr1-2*, and *etr1-1* in response to 0, 1, 10, and 100uL/L ethylene. The Number of cells starting from QC to the first cell present in the elongation zone in the cortex layer of the roots was counted with Optical Microscope (Nikon Eclipse 90i). Significant differences are shown by Asterisks. **(C)** Figure C shows the dose response of wild-type seedlings with 0, 0.1, 1, 10, and 100 uM ACC concentrations. Error bars indicate SE. Different letters show a significant difference by ANOVA.

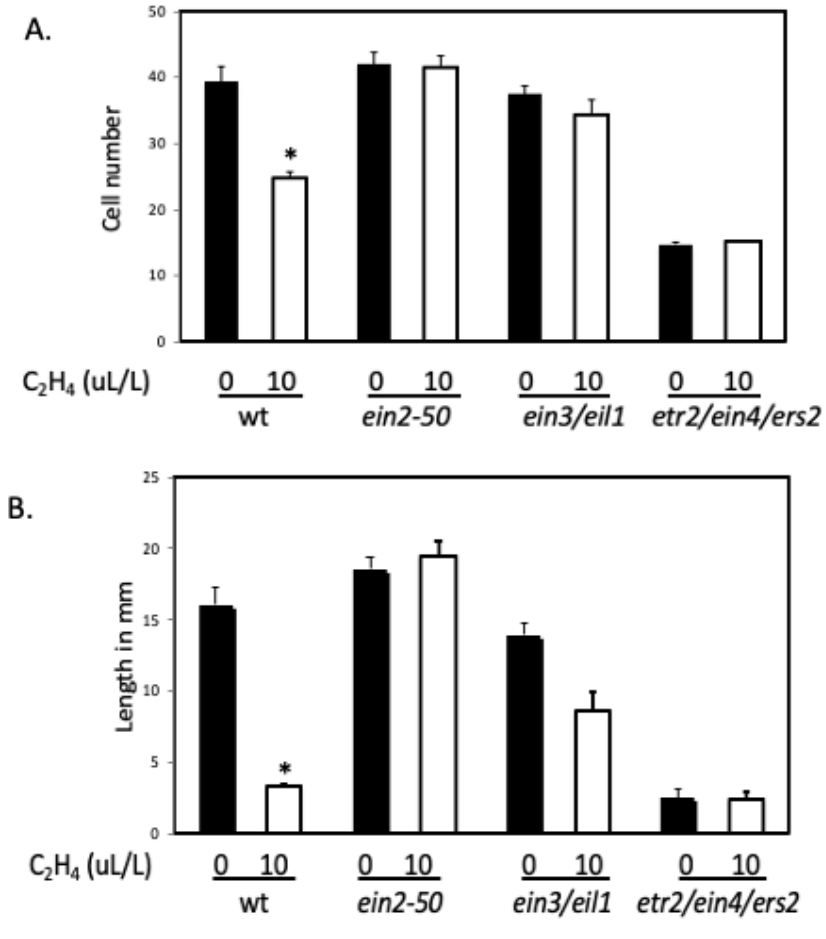

Figure 3.4: Additional Ethylene-Pathway Mutants Inhibit Cell Division at the RAM. Additional ethylene pathway mutants were grown for nine days under continuous light in the presence and absence of 10uL/L ethylene**.** RAM size was measured by counting the cortex layer cells of the proliferation zone, starting from the QC to the first cell present in the elongation zone. Root lengths were measured with ImageJ in mm. **(A)** RAM size of wild type, *ein2-50, ein3/eil1,* and *etr2/ ein4/ ers2*. **(B)** Root Length of wt, *ein2-50*, *ein3/eil1,* and *etr2/ein4/ers2* in mm. Error bars indicate SE. Asterisks show significant differences between mutants compared to wt (n = 15; $p < 0.05$).

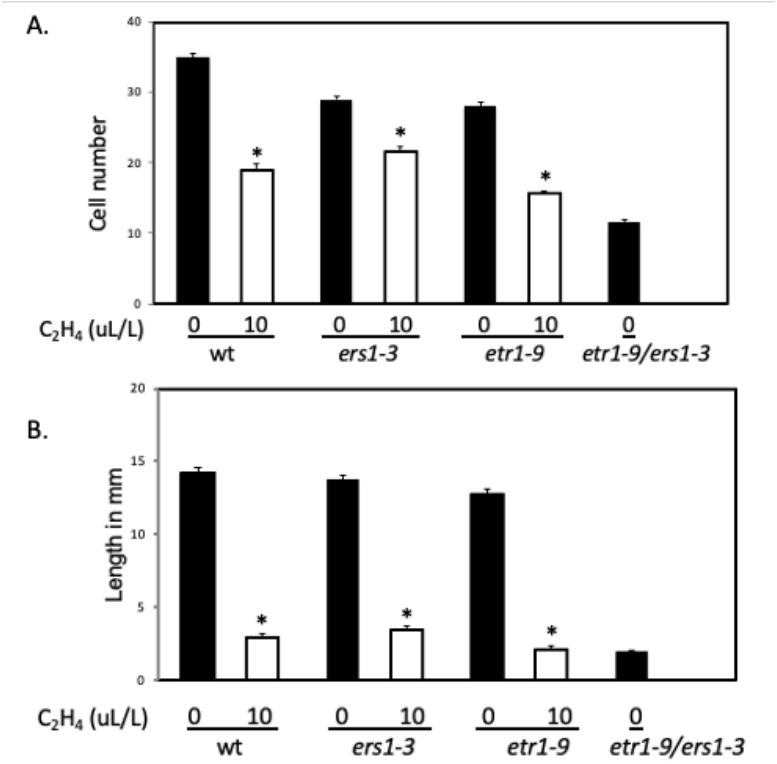

Figure 3.5: Cell number and Length of Additional Ethylene-Pathway Mutants. Additional ethylene pathway mutants were grown for nine days under continuous light in the presence and absence of 10uL/L ethylene**.** RAM size was measured by counting the cortex layer cells of the proliferation zone, starting from the QC to the first cell present in the elongation zone. Root lengths were measured with imageJ in mm. **(A)** RAM size of wild-type, *ers1-3/etr1-9, and etr1-9*, *ers1-3*. **(B)** Root length of wt, *ers3, etr1-9,* and *ers1-3/etr1-9* in mm. Error bars indicate SE. Asterisks show significant differences between mutants compared to wt ($n = 15$; $p < 0.05$).

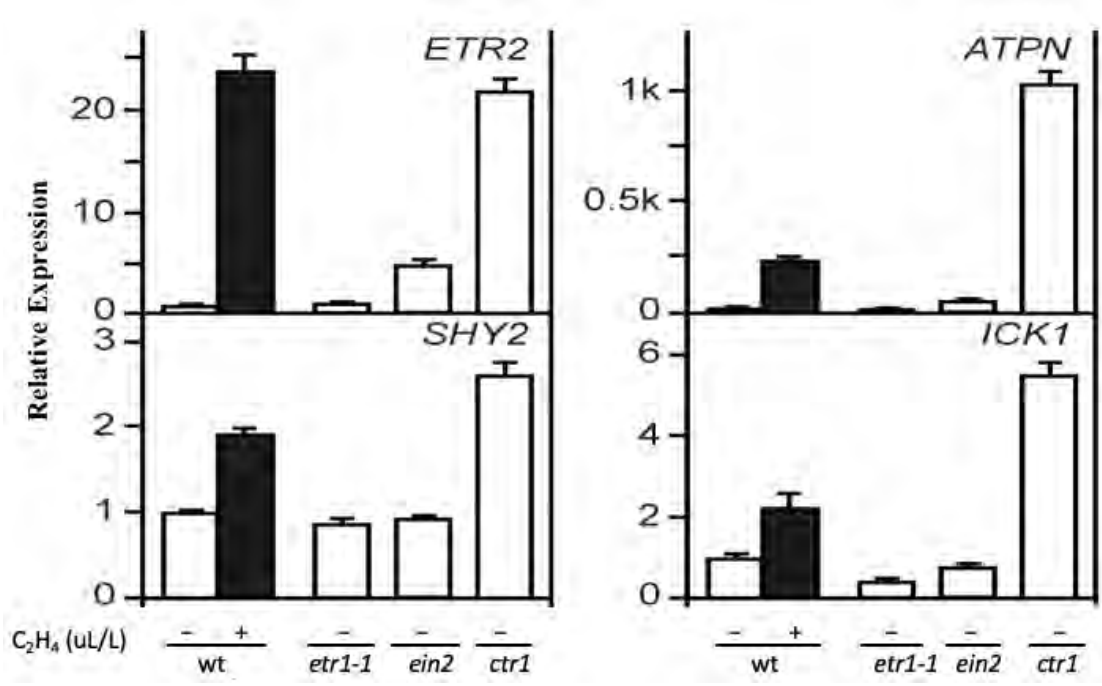

Figure 3.6: Molecular Analysis of Wild type and Ethylene Mutant Seedlings at RAM. 1mm root tips were obtained from the wild type, *etr1-1*, *ein2,* and *ctr1* mutants. Wild type and mutant seedlings were kept for nine days under continuous light in the (+) and (-) of 10uL/L of ethylene for growth. Gene expression was analyzed by qRT PCR. *Beta tubulin* was used as an internal control to normalize the expression data and is shown relative to wild-type untreated control. ETR2 and ATPN genes are positive control genes for ethylene response. Error bars indicate SE.

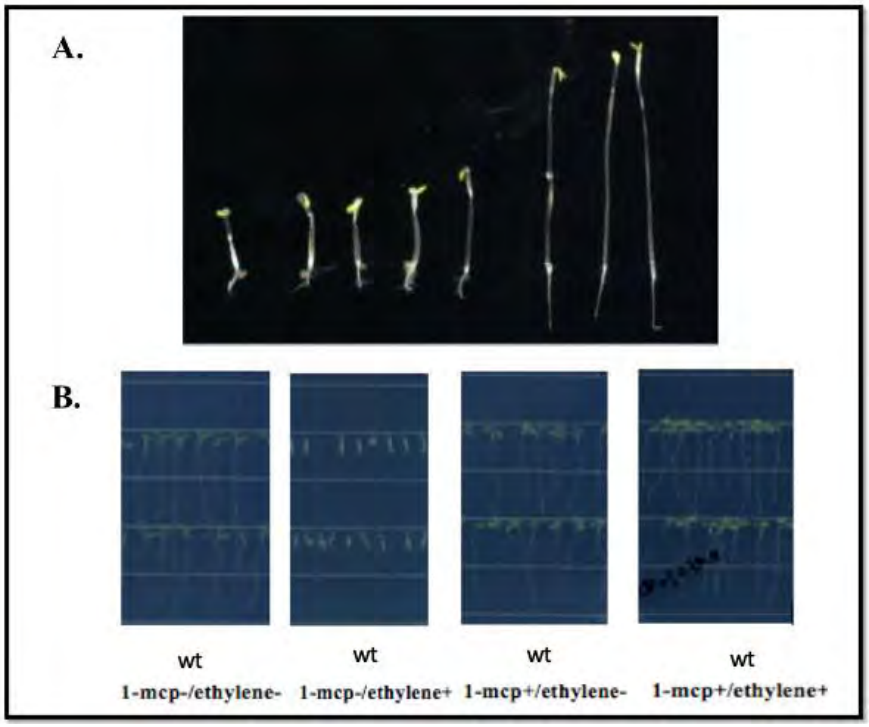

Figure 3.7: Phenotypic Representation of Wild type *Arabidopsis* Seedlings in (+) and (-) 1-MCP and Ethylene (A) Representative seedlings of 1-MCP dose response treated with 1 uL/L ethylene. Seedlings from left to Right are treated with 0nL, 1nL, 5nL, 10nL, 100nL, 1000nL, air (0uL/L ethylene and 0nL/L 1-MCP) and 1000nL 1-MCP only. All the seedlings were treated with 1uL/L ethylene before 1-MCP. Represented Seedlings show that increasing the dose of MCP blocks the effects of ethylene in a dose-response manner. **(B)** Phenotypes of wild-type *Arabidopsis* in the +/- of 10 uL/L ethylene and 10uL/L 1-MCP.

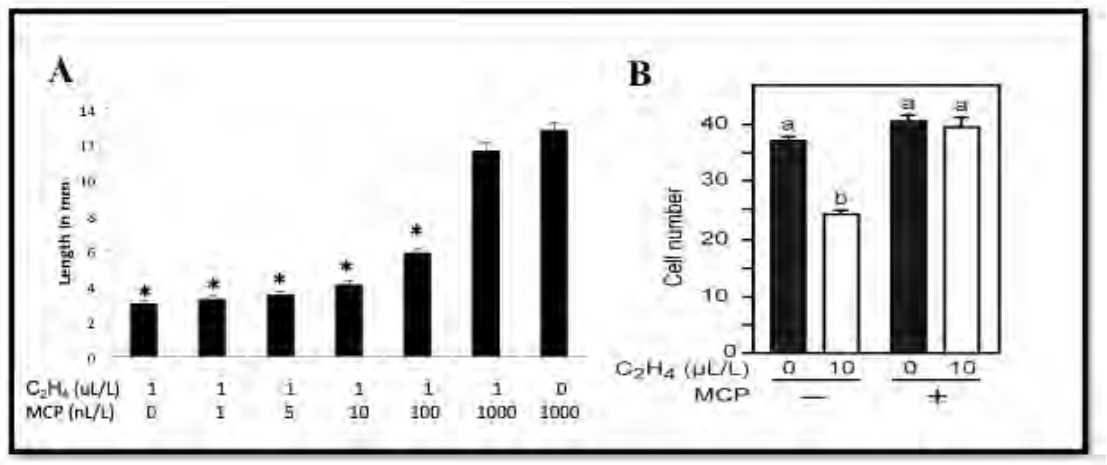

Figure 3.8:1-MCP Competitively Inhibits the Action of Ethylene. (A)Wild-type seedlings were grown in 1 uL L^{-1} ethylene with different quantities of 1-MCP in the dark for four days. The length was measured in mm by imageJ software. Data shows a gradual increase in the length of hypocotyl with increasing dose of 1-MCP. Each data shows the measurements of at least 20 seedlings ($n = 20$; $p < 0.05$) and error bars indicate SE. **(B)** Wild-type seedlings were exposed to 10 uL/L 1-MCP and 10 uL/L ethylene with controls grown in +/- 1-MCP to check the inhibitory action of 1-MCP on ethylene treatment. The number of cells starting from QC (quiescent center) to the first elongated cell in the cortex layer of roots was determined using Nikon Eclipse 90i Optical Microscope. Wild-type seedlings were exposed to 10 µL L^{-1} ethylene and 10 µL L^{-1} 1-MCP to determine the RAM size ($n = 10$; $p < 0.05$).

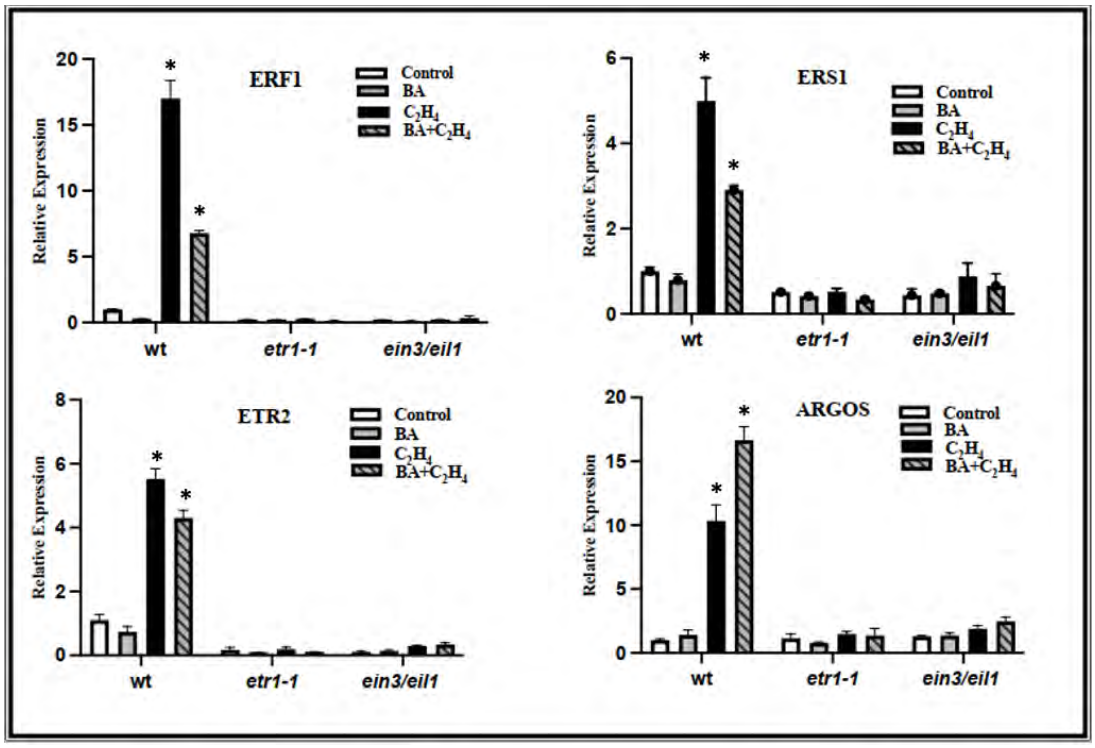

Figure 3.9: Competitive and Combinatorial Effect of Ethylene-Induced Genes in Etiolated Seedlings. Wild type, *etr1-1,* and *ein3/eil1* (ethylene insensitive mutant) seedlings were grown on MS media for 4 days in the dark. Gene expression of ethylene-induced genes (ERF1, ETR2, ERS1, and ARGOS) was examined to check the effect of ethylene, BA, and co-treatment of ethylene + BA by qRT-PCR. Expression of target genes was normalized with tubulin as an internal control and shown as relative to wild-type control (untreated). Error bars represent SE, and a significant difference was calculated by using One-way ANOVA with posthoc Tukey HSD Test Calculator for comparing multiple treatments using the reference of wild type (wt) for all four treatments among *etr1-1* and *ein3/eil1*. Asterisks show significant differences between mutants compared to wt (*, $P < 0.05$; **, $P < 0.01$).

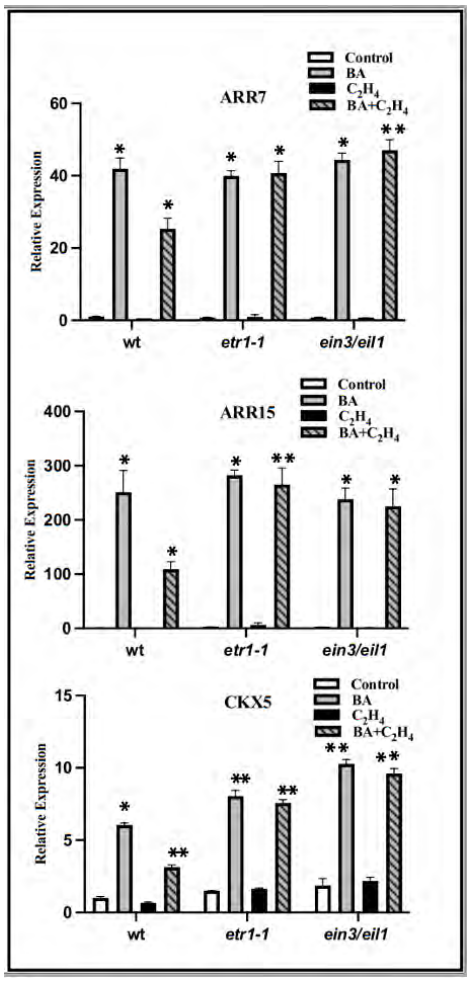

Figure 3.10: Co-Treatment Reduces the Transcript of Cytokinin-Induced Genes in Wild Type. wild type, *etr1-1,* and *ein3/eil1* (ethylene insensitive mutant) seedlings were grown on MS media for four days in the dark. Ethylene and BA were given in four different treatments to check the effect of single and combined treatment of BA and ethylene. 1 µL/L ethylene, 1uM BA & 1uL/L ethylene + 1uM BA together for two hours. Gene expression of cytokinin-induced genes (CKX5, ARR7 & ARR15) was studied with real-time-PCR. Expression of target genes was normalized with tubulin as an internal control and shown as relative to wild-type control (untreated). Error bars represent SE, and a significant difference was calculated by using One-way ANOVA with posthoc Tukey HSD Test Calculator for comparing multiple treatments using the reference of wild type (wt) for all four treatments among *etr1-1* and *ein3/eil1*. Asterisks show significant differences between mutants compared to wt (*, $P < 0.05$; **, $P < 0.01$).

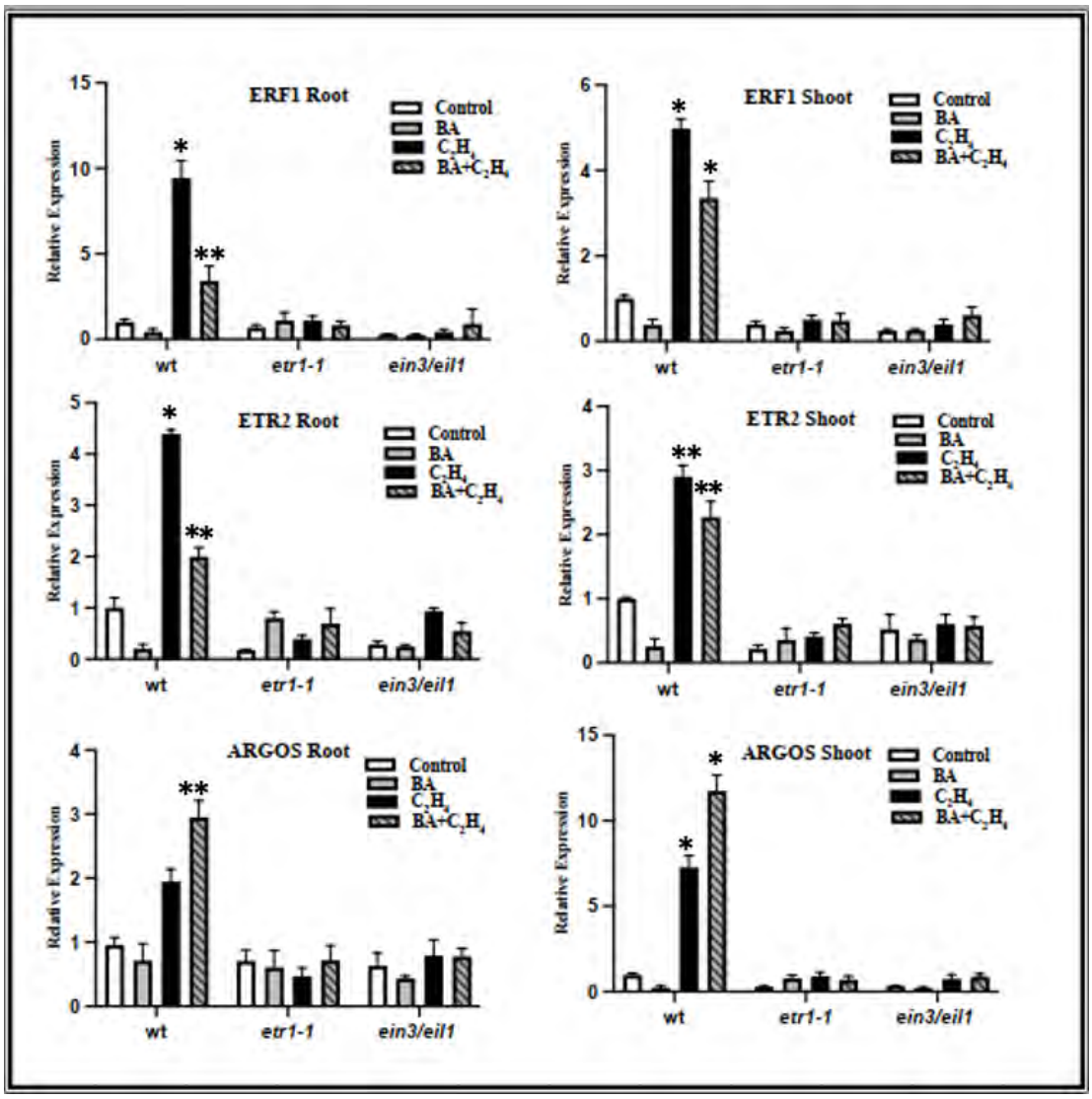

Figure 3.11: Gene Expression of Ethylene Induced Genes in Roots and Shoots. Wild type, *etr1-1* and *ein3/eil1* (ethylene insensitive mutant) Expression of (ERF1, ETR2, ERS1, and ARGOS) in shoot and root to check the effect of ACC, BA, and co-treatment of ACC + BA by qRT-PCR. Expression of target genes was normalized with tubulin as an internal control and shown as relative to wild-type control (untreated). Error bars represent SE, and a significant difference was calculated by using One-way ANOVA with posthoc Tukey HSD Test Calculator for comparing multiple treatments using the reference of wild type (wt) for all four treatments among *etr1-1* and *ein3/eil1*. Asterisks show significant differences between mutants compared to wt (*, $P < 0.05$; **, $P < 0.01$).

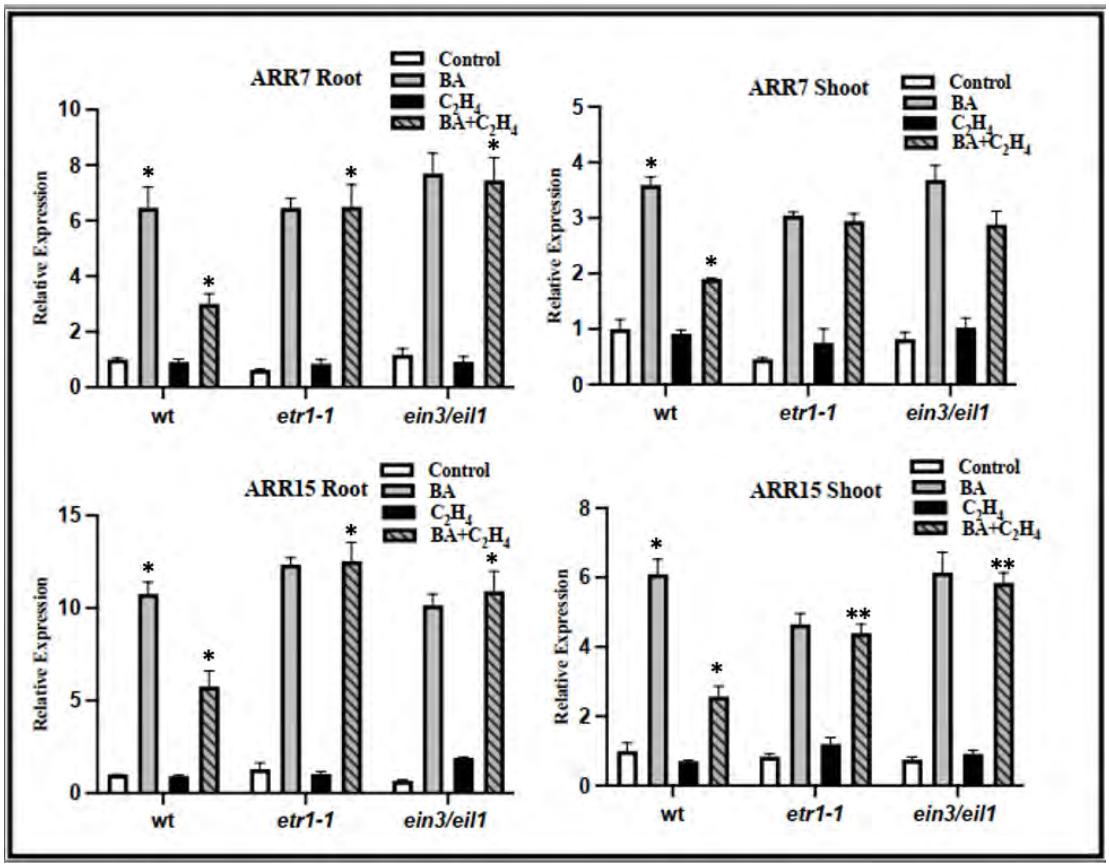

Figure 3.12: Cytokinin-Induced Genes Show Competitive Effect in Response to Co-Treatment. wild type, *etr1-1,* and *ein3/eil1* (ethylene insensitive mutant) seedlings were grown for seven days in constant illumination. Cytokinin-induced genes (ARR7 and ARR15) were analyzed in root and shoot to check the effect of ACC, BA, and co-treatment of ACC + BA by qRT-PCR. Expression of target genes was normalized with tubulin as an internal control and shown as relative to wild-type control (untreated). Error bars represent SE, and a significant difference was calculated by using One-way ANOVA with posthoc Tukey HSD Test Calculator for comparing multiple treatments using the reference of wild type (wt) for all four treatments among *etr1-1* and *ein3/eil1*. Asterisks show significant differences between mutants compared to wt (*, $P < 0.05$; **, $P < 0.01$).

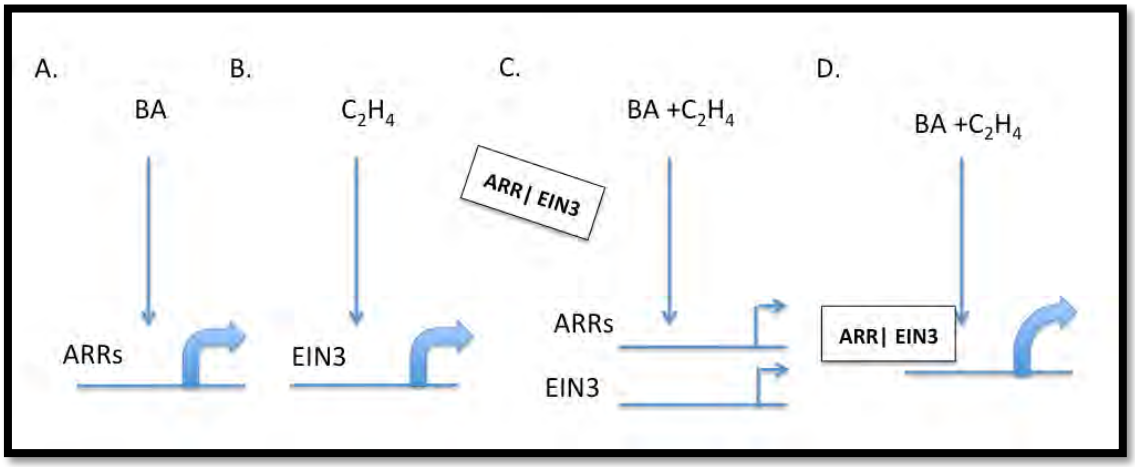

Figure 3.13: Potential Competitive and Combinatorial Effect of Ethylene and Cytokinin. Four possibilities of gene expression in response to the treatments conducted in our study. Fig A shows increased expression of cytokinin response genes in response to cytokinin. B shows increased expression of ethylene response genes in response to ethylene. C shows the subtractive effect on gene expression in response to concurrent ethylene and cytokinin treatment. D shows the additive expression in response to concurrent ethylene and cytokinin treatments.

3.2 Negative Regulation at Root Apical Meristem

To find the effect of ethylene on root apical meristem, green seedlings were grown for nine days under continuous illumination at 24°C. Continuous illumination increases the growth of plants as it gets light to carry out photosynthesis for 24 hours. The phenotype of wild-type seedlings and ethylene mutants *etr1-1, ein 2-50* and *ctr1-2* (Fig.3.2). *etr1-1* is a GOF ethylene insensitive mutant, this mutation has lost its ability to sense ethylene making it insensitive to ethylene. *ctr1-2* is a LOF ethylene constitutive mutant, with the ability of enhanced ethylene signaling and biosynthesis even in ethylene's absence. It is constitutively showing the ethylene-responsive phenotype. Loss of function mutant *ctr1-2* exhibits a constitutive ethylene-responsive phenotype (Hall and Bleecker, 2003). *ctr1-2, ein 2-50, and etr1-1* were treated with 10 uL/L ethylene in a closed container, and control wt was kept in the air under the same conditions for the determination of ethylene's effect on cell proliferation of the Root Apical Meristem (RAM). The application of exogenous

ethylene greatly reduced the root size in the wild type forming a small root as compared to the control. Ethylene insensitive mutants *ein 2-50* and *etr 1-1* showed no significant reduction in the root size in response to ethylene as compared to the untreated (Fig. 3.2). Missense mutation in the ETR family receptors of ethylene makes them insensitive to ethylene. No ethylene-responsive phenotype was observed in *etr1, etr2, ers2,* and *ein4* (Hall and Bleecker, 2003). *ctr1-2* is a constitutive mutant and showed a small root size in both treated and untreated. The root size of *ctr1-2* is equal to the root size of ethylene-treated wild-type seedlings. Ethylene-treated *ctr1-2* showed a little reduction in the root size as compared to the untreated (Fig 3.2). This indicates *ctr 1* independent ethylene response in these seedlings. *ctr1-2* exhibits a small meristem with a significantly reduced number of cells in the meristem (Thomann et al., 2009). Exogenous ethylene treatment or ACC treatment in *Arabidopsis* inhibits the primary root growth. *ctr1-2* and *eto 1,* ethylene overproducer enhances ethylene biosynthesis and signaling show short primary root. Ethylene-insensitive mutants such as *ein 2*, *etr 1*, and *ein3/eil1* do not show the inhibitory effects of ethylene on root growth (Qin and Huang, 2018).

Microscopic analysis of RAM by counting the number of cells present in the cortex layer indicates the size of the root meristem (Perilli and Sabatini, 2010). Smaller roots have a smaller number of cells present in the meristematic zone of the root and larger roots have a greater number of cells present. Cells of the cortex layer are preferred over the epidermis layer because they remain constant among different roots. The epidermal layer consists of two types of cells i-e, hair and non-hair. Hair cells of the epidermis layer divide more rapidly, this makes it variable among different roots as compared to the constant number of cortex layer cells (Perilli and Sabatini, 2010). Staining the roots with propidium iodide gave a clear picture for visualization with the help of a Nikon A1 Confocal Microscope. The number of cells in wild type treated with 10 uL/L ethylene was greatly reduced with reduced RAM size as compared to the RAM size of untreated seedlings (Fig. 3.3 A). RAM size was determined by counting the cells starting from the QC to the first cell present in the elongation zone. This is an established method used for the quantification of anticlinal divisions occurring in the root apical meristem (Perilli and Sabatini, 2010).

Untreated wild type exhibited 33 cells in the meristem and ethylene-treated seedlings exhibited 20 cells in the meristem with the reduction in RAM size (Fig 3.3 A).

Similarly, the RAM size of wt, *etr1-1,* and *ctr 1-2* was examined under the microscope after treatment with different ethylene concentrations. *etr 1-1,* ethylene insensitive mutant showed less response to ethylene and exhibited larger RAM as compared to the untreated wild type. It showed only a 20% decrease in the number of cells in response to 100 uL/L ethylene as compared to the untreated (42 cells in untreated and 34 in the treated). The RAM size of constitutive mutant *ctr1-2* was the same as the RAM size of ethylene-treated wild type at 100uL/L. Wild-type seedlings showed a gradual decrease in the RAM size with increasing ethylene concentrations from 0, 1, 10, and 100 uL/L. Number of cells in the wild type reduced to 14 in 100uL/L ethylene as compared to 33 cells in the untreated showing a 58% reduction in the RAM size (Fig. 3.3B).

ACC, an ethylene biosynthetic precursor, was used in a dose-response manner to find the inhibitory response of ethylene in RAM. Wild-type seedlings showed a gradual decrease in the RAM size with increasing doses of ACC (Fig 3.3 C). Root apical meristem activity was inhibited by ACC same as exogenous ethylene. These results indicate that ethylene inhibits the root apical meristem by inhibiting the proliferation of cells. ACC was used in place of ethylene in a previous study of RAM (Růzicka et al., 2007), Administration of the gaseous plant hormone ethylene was carried out by using its precursor ACC. ACC is the precursor of ethylene decreased cell elongation in *Arabidopsis thaliana* roots (Markakis et al., 2012).

3.3 Effect of Ethylene on Additional Ethylene Insensitive and Constitutive Mutants at RAM

To make a better understanding of how RAM size is regulated by ethylene, some additional ethylene mutants were examined. Receptors of the ethylene signaling pathway are divided into two subfamilies according to their evolutionary relationships and features. Subfamily 1 consists of ETR1, ERS1, and receptors of subfamily 2 are ETR2, ERS2, and EIN4 (Binder et al., 2012). *esr1-3* and *etr1-9* are LOF mutants of the subfamily 1. Subfamily 2 LOF mutant is a triple mutant *etr2/ein4/ers2* used in the study (Fig.3.5 & 3.4). *ein2-50* is a downstream insensitive mutant. *ein3/eil1* is a transcription factor mutant (Fig.3.4). LOF mutation in the receptors leads to the constitutive ethylene signaling in plants as receptors of the ethylene pathway function as negative regulators of the pathway (Hua and Meyerowitz, 1998, Qu et al., 2007).

Our results tell that *ein3/eil1* and *ein2-50* being insensitive mutants were very less responsive to ethylene. *ein2-50* is highly insensitive to ethylene. The RAM size of *ein2-50* is larger than the RAM size of wild type in air. This is because *ein2-50* is highly insensitive to ethylene and it also blocks the endogenous ethylene produced in plants. *ein3/eil1* is a partially insensitive mutant to ethylene. *etr2/ein4/ers2* triple constitutive ethylene mutant shows a smaller number of cells in the cortex layer like *ctr1-2* at 10uL/L of ethylene and exhibits a smaller RAM (Fig.3.4).

etr1-9 and *ers 1-3* unlike other mutants respond to ethylene the same as the wild type. RAM size of *etr1-9* and *ers 1-3* is similar to wild type; *etr1-9* is a little more sensitive to ethylene as compared to *ers1-3* showing more reduction in RAM size. *etr1-9 / ers1-3* double mutant was recognized in the segregation of *etr1-3/ers1-3*$^{-/+}$ background based on its constitutive ethylene signaling phenotype was shown (Qu et al., 2007). *etr1-9/ers1-3* displayed a decreased RAM size same as found in the *ctr1-2* mutant. In the presence of ethylene, the phenotypic characterization of the *etr1-9 / ers1-3* mutant was not possible because the ethylene response was indistinguishable (Fig 3.5).

Altogether, this data shows that ethylene plays in negative regulation of cell proliferation in the root apical meristem, by using the canonical pathway of ethylene that consists of five ethylene receptors subdivided into two subfamilies, CTR1, EIN2, and the EIN3/EIL which are the transcription factors of pathway.

(Thomann et al., 2009), observed a reduced meristem in Cullen mutants and found the role of ethylene in reducing the meristem size. They generated mutant combinations of Cullen with ethylene-insensitive mutants *etr1-1* and *ein2*. They found that ethylene-insensitive mutants reverted the short meristem observed in the Cullen mutation. These results indicate the involvement of ethylene in inhibiting the proliferation of cells in the RAM.

Different hormones are responsible for the transition of cells from division to elongation/differentiation in the RAM. Auxin and cytokinin are well-known players in this transition but ethylene, gibberellins, and brassinosteroids are also key components for the transition of dividing cells to the elongated cell in the meristem of root (Bizet et al., 2014). Ethylene receptor ETR1 may function through multistep phosphorelay to switch on the cytokinin signaling via activation of AHK, AHPs, and ARRs to inhibit the proliferation of cells at root apical meristem (Chen et al., 2022).

3.4 Gene Expression Analysis of Wild Type and Ethylene Mutants at Root Apical Meristem.

After Counting the number of cells in the proliferation zone in response to ethylene and identifying the role of ethylene in reducing the RAM size. We examined the role of ethylene at the molecular level in wild-type and ethylene mutants in the RAM. Gene expression of wild type, *etr 1-2*, *ein 2-50* (ethylene insensitive mutants), and *ctr 1-2* (constitutive mutant) was examined using qRT PCR. Seedlings were grown in continuous light for nine days in the presence and absence of 10uL/L ethylene. 1 mm size of roots was cut to obtain the RNA present in the proliferation zone of RAM so that our results do not interfere with the molecular expression of genes in the elongation/differentiation region of the root. Gene expression of *ETR1, ATPN, SHY2*, and *ICK1* was determined in wild type in response to ethylene and was compared to the insensitive mutants of ethylene *etr1-1* and *ein2-50* and constitutive mutant *ctr 1-2*. *ETR1* and *ATPN* (*Arabidopsis* PEROXIDASE N) were used as positive controls of ethylene response. *ETR1* and *ATPN* are known genes for ethylene response and are induced with ethylene (Hall et al., 2012). High expression of *ETR1* and *ATPN* was observed in the wild type in response to ethylene and the constitutive ethylene mutant *ctr1-2.* It confirmed the effectiveness of applied ethylene. Expression of two other genes *SHY2* and *ICK1* was checked to confirm their involvement in inhibiting RAM size. SHY2 is the point of convergence in Auxin – Cytokinin signaling at RAM. SHY2 encodes IAA3 (Indole-3 Acetic acid Inducible3), an auxin repressor to halt the proliferation of cells and force the cells to elongation /differentiation zone. Cytokinin induces SHY2 at RAM to negatively regulates cell proliferation. This is a well-studied cytokinin-auxin crosstalk at RAM (Ioio et al., 2008). ICK1 negatively regulates the cell cycle by encoding CDK (cyclin-dependent kinase) inhibitors. Induction of ICK inhibits the progression of the cell cycle and decreases the rate of cell proliferation in the shoots and roots (Cheng et al., 2013, Beemster et al., 2005). Gene expression of *SHY2* and *ICK1* was elevated in wild type in ethylene's response and *ctr1-2*. High expression of *SHY2* in response to ethylene indicates that ethylene induces SHY2 in RAM to inhibit proliferation. SHY2 is the point of convergence for ethylene signaling in RAM same as it is for cytokinin. High expression of *ICK1* in ethylene-treated wildtype and *ctr1-2* confirms the inhibition of cell proliferation by ethylene (Fig.3.6). Cytokinin is well studied in the negative regulation of cell proliferation similar to

ethylene (Ioio et al., 2008, Werner et al., 2003). It is also known to induce ethylene biosynthesis and this is done by stabilizing the ACS (1-aminocyclopropane-1-carboxylate synthase) that is involved in ethylene biosynthetic pathways, and this process also facilitates the cytokinin to inhibit the cell expansion (Chae et al., 2003, Růžička et al., 2009, Vogel et al., 1998). Induction of ethylene by cytokinin leads to an added role for ethylene to inhibit cell proliferation at root meristem, opposite to an earlier study that cytokinin functions independently of ethylene in repressing proliferation (Růžička et al., 2009).

3.5 1-MCP Act as a Competitive Ethylene Inhibitor

Another well-known pharmacological approach was used to study the inhibitory effect of ethylene. 1-MCP is a known Pharmacological agent that competitively inhibits ethylene. We checked the phenotype of wild-type seedlings treated with 1-MCP to check the recovery of inhibitory effects of ethylene by 1-MCP in a dose-response manner. Four days old wild-type etiolated seedlings treated with 1uL/L ethylene. 1-MCP was applied to the ethylene-treated etiolated seedlings in different doses starting from 1nL/L, 5nL/L, 10nL/L, 100nL/L 1000nL/L, air, and 1000nL/L 1-MCP only. The phenotype of ethylene and 1-MCP treated wild-type etiolated seedlings showed that as the dose of 1-MCP increased ethylene's inhibitory effect was decreased (Fig. 3.7A). (+ethylene/-1-MCP) wild type seedlings showed a clear ethylene response phenotype, characterized by thick short hypocotyl, inhibition of root formation, and exaggerated apical hook formation (Fig.3.7B). 1-MCP recovered the ethylene's inhibitory effect on seedlings in a dose-response manner (Fig.3.7A). The root length of seedlings was measured with imageJ. The root length of seedlings increased with the increasing dose of 1-MCP (Fig 3.8A). We also used 1-MCP to check ethylene's inhibitory effect on cell division at the root apical meristem. Nine days old wild type seedlings were grown in light in the presence/absence of 10uL/L ethylene and treated with and without 10 uL/L 1-MCP to check the recovery of the inhibitory effect of ethylene at RAM. The cell number in RAM of ethylene-treated seedlings in the absence of 1-MCP was reduced to 24 as compared to the cell number of untreated seedlings(-ethylene/-1-MCP). Whereas seedlings treated with 1-MCP both (+ethylene/+1-MCP) and (-ethylene/+1-MCP) exhibited larger RAM as compared to the untreated (-ethylene/-1-MCP) seedlings. This shows that 1-MCP not only inhibits the exogenously applied

ethylene but also inhibits the endogenous levels of ethylene. This experiment further confirms the inhibitory effects of ethylene at RAM (Fig.3.8B).

1-MCP is considered to be the specific and competitive inhibitor of ethylene, as it can bind to the ethylene receptor with a higher affinity (Hall et al., 2000, Sisler, 2006). To inhibit the ethylene binding and its synthesis some other pharmacological agents like aminoethoxyvinylglycine (AVG) and silver (Ag) were also used and showed a profound impact on the activity of auxin (Soeno et al., 2010, Strader et al., 2009).

(Santisree et al., 2011) stated that penetration of roots in the soil during seed germination of tomato requires ethylene. Application of 1-MCP during seed germination failed to penetrate roots in the soil. 1-MCP is the inhibitor of ethylene and inhibited the penetration of roots.

3.6 Co-treatment of Cytokinin and Ethylene Induces Competitive and Combinatorial Effect at Transcript Level

To get the insight of cytokinin and ethylene interaction we checked the wild-type seedlings of *Arabidopsis thaliana* and insensitive mutants of ethylene (*ein3/eil1* and *etr1-1*). We checked the effects of cytokinin and ethylene with a single application of ethylene and cytokinin and the effects of co-treatment of cytokinin and ethylene on both wild-type seedlings of *A. thaliana* and insensitive mutants of ethylene. We examined the gene expression of different ethylene-induced genes and cytokinin-induced genes in wild-type and insensitive mutants of ethylene *etr1-1* and *ein3/eil1*. As ethylene-insensitive mutants should not respond to ethylene, the induction in the

Ethylene-responsive genes in ethylene-treated samples will not be observed in ethylene-insensitive mutants as compared to wild-type. No induction in ethylene-insensitive mutants demonstrates the involvement of ethylene in inducing the transcript levels in the wild type. All wild-type and ethylene-insensitive mutants were grown on MS media in the dark for four days. Two hours of a single treatment of cytokinin and ethylene and co-treatment of cytokinin and ethylene were given. DMSO was treated as a control. Ethylene induced genes *ERF1, ETR2, ERS1, ARGOS* (Fig. 3.9) and cytokinin induced genes *ARR7, ARR15* and *CKX5* (Fig. 3.10) were analyzed by qRT PCR. The same set of treatments, i.e., single treatments of ethylene and cytokinin and co-treatment of ethylene and cytokinin on wild-type and ethylene-insensitive mutants were used to check the effects of treatments on light-grown

seedlings. The interaction of ethylene and cytokinin and its outcome on etiolated seedlings and shoots and roots of light-grown seedlings was observed and compared. Plants go through different developmental stages when grown in light and dark. The phenomenon of growing plants in the dark is known as etiolation with some special features, leaves produce yellow color due to carotenoids, and developmental stages include elongated hypocotyl with a hook and cotyledon which is underdeveloped (Xiong et al., 2017). The growth of etiolated plants is known as skotomorphogenesis. Etiolated seedlings usually have a small leaf area and dwarf plants. As soon as plants receive light, they transit from skotomorphogenesis to photomorphogenesis and the process of light-grown seedlings is known as de-etiolation. Plant shoots undergo physiological, morphological, and biochemical changes in light (Xiong et al., 2017). Ethylene treatment shows different outcomes in etiolated and light-grown seedlings. Etiolated seedlings respond to ethylene with distinct features known as a triple response. The triple response is characterized by short and thick hypocotyl with an exaggerated apical hook. A Triple response is not seen in the ethylene-insensitive mutants. Light-grown seedlings respond differently from etiolated seedlings to ethylene with increased length of hypocotyl, opening of apical hook, and expanded cotyledons (Harkey et al., 2019). Interaction of cytokinin and ethylene was observed in both etiolated and light-grown seedlings (root and shoot) to check if they have different outcomes.

Ethylene-induced genes *ERF1, ETR2*, and *ERS1* were induced in ethylene's response in wild-type etiolated seedlings. Single treatment of cytokinin did not induce the expression of ethylene response genes in the wild type. But the concurrent treatment of cytokinin and ethylene showed a subtractive effect in the expression of *ERF1*, *ETR2*, and *ERS1* as compared to their level of induction in a single treatment of ethylene. This subtractive effect is due to the interaction of cytokinin with ethylene. Whereas *ARGOS* another ethylene-induced gene showed an additive effect in response to the concurrent treatment of ethylene and cytokinin (Fig.3.9). *ARGOS* is an ethylene response gene that acts as a negative regulator of ethylene. It binds to the ethylene receptors and blocks ethylene signaling (Dubois et al., 2018). Cytokinin-induced genes *ARR7* and *ARR15* were also checked in response to single treatments of ethylene and cytokinin and concurrent treatment of cytokinin and ethylene in wild-type and insensitive mutants of ethylene *etr1-1* and *ein3/eil1. ARR7* and *ARR15* are cytokinin

response genes that belong to type A response regulators. Type A response regulators act as transcriptional repressors of cytokinin signaling genes (Jeon et al., 2010). CKX, cytokinin oxidase is a negative regulator of cytokinin signaling and is responsible for the destruction of cytokinin. It acts to regulate the levels of cytokinin (Chen et al., 2020). Gene expression of *ARR7, ARR15,* and *CKX5* showed the subtractive effect in response to the concurrent treatment of ethylene and cytokinin. Ethylene reduced the expression of *ARR7, ARR15,* and *CKX5* in concurrent treatment with cytokinin (Fig. 3.10).

(Shi et al., 2012) reported that cytokinin signaling is modulated by ethylene. Transcription factors of cytokinin signaling pathway were increased in the double mutant *ein3 eil1* as compared to wild type; ARR7 and ARR15 were found to be higher in the ethylene mutant in comparison to wild-type plants in cold induction. Reduced expression in the wild type was suppressed by ethylene. These results in response to cold induction revealed that the EIN3 transcription factor of the ethylene pathway suppresses these ARRs, which was then confirmed by the use of direct binding assays. Treatment of plants with ethylene precursor ACC showed that the expression profile of these ARRs was dramatically reduced and its induction through cold temperatures was also blocked, and thus the freezing tolerance of plants was reduced hence suggesting that transcription factors of cytokinin ARR5, ARR7, and ARR15 participate and interact with ethylene to regulate the freezing response of plants.

Insensitive mutants of ethylene *etr1-1* and *ein3/eil1* are insensitive to ethylene so cytokinin-induced genes in response to BA were induced in these mutants same as they were induced in wild-type seedlings in response to the single treatment of cytokinin. In wild-type seedlings, we saw a reduction in the transcript level of *ARR7, ARR15*, and *CKX5* when co-treated with ethylene and cytokinin but this reduction was not seen in ethylene insensitive mutants in response to the co-treatment. This shows that there is an involvement of ethylene with cytokinin that causes a reduction in the transcript level (Fig.3.10).

Cytokinin is known to induce the biosynthesis of ethylene since old times. Cytokinin treatments cause morphological and developmental changes in plants similar to the triple response of ethylene. It also induces and stabilizes different levels of ACS post-transcriptionally (Zdarska et al., 2015).

Cytokinin and ethylene may interact with each other through MSP (multistep phosphorelay). Yeast two-hybrid analysis showed the interaction of ETR1 with AHP1 and 3 and with ARR4 through AHPs. Through the MSP pathway ethylene induces type B ARRs and type B ARRs also function in ethylene signaling other than the cytokinin signal pathway (Zdarska et al., 2015).

Cytokinin and ethylene interaction are known at the level of biosynthesis as both cytokinin and ethylene induce the biosynthesis of each other. Cytokinin induces and stabilizes the levels of ACS but they also interact with each other at the level of signal output. EIN3 interacts with ARRs to suppress the cytokinin signaling by suppressing the expression of ARRs (Chen et al., 2022).

3.7 Analysis of Ethylene and Cytokinin-Induced Genes in Roots and Shoots with Ethylene and Cytokinin Treatments.

The interaction of cytokinin and ethylene was also analyzed in the green seedlings. As already mentioned above that plant goes through different phases of development in etiolated and green seedlings. Phytohormones may also function differently in light-grown seedlings than in etiolated seedlings. In light-grown seedlings roots and shoots were separated to check the interaction of hormones in them separately. The crosstalk of hormones in the root differs from the crosstalk in the shoots. As cytokinin is known to inhibit root but it induces shoot formation. Crosstalk of auxin and cytokinin differ in root and shoot. In tissue culture experiments, ratios of cytokinin and auxin hormones decide the formation of a shoot or root from the callus (Kurepa and Smalle, 2022). Another purpose of separating root and shoot was to check if ethylene and cytokinin interact differently in root and shoot. As we already know that both hormones inhibit root formation but act antagonistically in the shoot. Cytokinin promotes shoot formation whereas ethylene inhibits shoot (Dubois et al., 2018).

Wild type and insensitive mutants of ethylene *etr1-1,* and *ein3/eil1* of *Arabidopsis* were grown for 7 days in light and were treated with cytokinin, ethylene, and concurrently with ethylene and cytokinin, and DMSO was used as a control for two hours. Ethylene response genes *ETR1, ERS2, ERF1*, and *ARGOS* were checked in both shoot and root of wild type and ethylene insensitive mutants to check the effect of concurrent treatment of ethylene and cytokinin. We observed similar results in roots and shoots as we observed in etiolated seedlings. Concurrent treatment showed

subtractive effects in *ERF1*, *ERS2,* and *ETR1* gene expression in wild-type seedlings. An additive effect in the gene expression of ARGOS was observed in both shoot and root (Fig.3.11). Cytokinin-induced genes *ARR7* and *ARR15* were also checked in root and shoot. We also observed similar results of cytokinin-induced genes as observed in etiolated seedlings. *ARR7* and *ARR15* were induced in response to cytokinin in wild-type and ethylene-insensitive mutants. Concurrent treatments showed a subtractive effect in wild type but not in ethylene-insensitive mutants. *ein3/eil1* being partially insensitive to ethylene shows a little but insignificant induction of ethylene (Fig.3.12).

The complicated interactions of ethylene and cytokinin are broadly studied. Stabilization of ACS5, a biosynthetic enzyme of ethylene has been reported to be done by cytokinin, which results in an increased production of ethylene (Chae et al., 2003, Hansen et al., 2009, Vogel et al., 1998). It is thought that ethylene mediates several responses of cytokinin (Cary et al., 1995, Golan et al., 1996, Tanaka et al., 2006). Type-B ARR2 acts downstream of the ethylene signaling pathway with the ETR1 receptor and functions as a two-component signaling (Hass et al., 2004). Bioinformatics study revealed that type-A ARR genes, such as ARR5, ARR7, and ARR15, are the downstream targets of EIN3 (Hass et al., 2004).

To test the direct binding of EIN3 with type A ARR genes EMSA (electrophoretic mobility shift assays) was performed in vitro. The recombinant EIN3 protein was used which consists of a DNA binding domain expressed in *E. coli* as a glutathione S-transferase (GST) fusion protein and was then purified (Chen et al., 2009, Cary et al., 1995, Chae et al., 2003, Golan et al., 1996, Hansen et al., 2009, Hass et al., 2004, Tanaka et al., 2006, Vogel et al., 1998). EIN3 contains the binding sites and thus directly binds to the promoters of the CBF1-CBF3, ARR5, ARR7, and ARR15 genes in vitro. ChIP assays together with these results, proposed that CBF1-CBF3 and ARR5, ARR7, and ARR15 are the direct targets of the EIN3 transcription factor.

EIN3/EIL1 functions as transcription factors present in the nucleus in the ethylene signaling pathway and binds to EIN3 binding sites (EBSs) present in the promoters of target genes (Chen et al., 2009, Kosugi and Ohashi, 2000, Zhang et al., 2011, Zhong et al., 2009).

Our results suggest that ethylene signaling functions in the same manner in etiolated seedlings and roots and shoots of light-grown seedlings. Light conditions do not affect

the signaling output of ethylene. Normal ethylene signaling requires EIN2 and CTR1 in all types of light conditions. CTR1 mutations show constitutive ethylene response in shoot and root in both light and dark conditions. Ethylene-insensitive mutants remain insensitive to ethylene in both light and dark. Ethylene signaling in roots is independent of light (Harkey et al., 2019). Our data suggest that the outcome of ethylene and cytokinin interaction is the same in the roots and shoots of light-grown seedlings and etiolated seedlings. The interaction of ethylene and cytokinin suppresses the outcome of each other. ACC and ethylene inhibit root elongation in both seedlings grown in dark and light (Rahman et al., 2002). The outcome in response to this interaction is not that profound in plant development and growth. We already discussed that inhibition of proliferation in root apical meristem by ethylene and cytokinin are independently operated. Ethylene partially activates cytokinin signaling via the histidine kinase domain of ETR 1 recruiting ARRs.

3.8 Conclusions of Part A

We found that ethylene inhibits cell proliferation at the RAM by using different ethylene insensitive and ethylene constitutive mutants. The reduced size of the Root Apical Meristem and decreased cell count in the Meristem in the presence of ethylene showed that ethylene is involved in the negative regulation of cell division at the Root Apical Meristem. Ethylene inhibits root apical meristem in the same way as cytokinin. Ethylene inhibits cell division by inhibiting the auxin at the root apical meristem through the action of SHY2. SHY2 is the repressor of auxin and is stimulated by ethylene at the root apical meristem to inhibit cell division and promote cell differentiation. Ethylene inhibits RAM the same as cytokinin through SHY2. Cytokinin antagonizes with auxin by using the same point of convergence used by ethylene, which is SHY2. Both hormones, ethylene and cytokinin use SHY2 to inhibit auxin at root apical meristem. Ethylene inhibits root apical meristem independent of cytokinin via its canonical linear pathway. We found from our real-time data described above that ethylene uses the same point of convergence as cytokinin which is SHY2 involved in the inhibition of cell proliferation at RAM. There might be some interaction between ethylene and cytokinin. Concurrent treatment of ethylene and cytokinin gave two types of gene expression, subtractive and additive. We proposed a model (Fig.3.1) based on this interaction that ethylene operates via multi-step phosphorelay to cause the interaction of EIN3 with ARRs of the cytokinin signaling

pathway. EIN3 has binding sites for ARRs. Two possibilities can be assumed for this reduction; the interaction of ethylene and cytokinin transcription factors Ein3 and ARRs causes sequestration and thus are not available for the binding to the promoter sequence. Less number of free transcription factors bind to the promoter and cause a decrease in the transcription level. Or the induction of Ein3 and ARRs recruits the EBF and KMD for the degradation of these transcription factors. Whereas induction of ARGOS, which is also the ethylene, induced gene showed increased expression in response to the co-treatment of ethylene and cytokinin in the wild type. Increased expression of some genes can be due to the interaction of Ein3 and ARRs and binding to a new promoter in response to the co-treatment of cytokinin and ethylene as seen in ARGOS. ARGOS has not been induced in ethylene-insensitive mutants, which indicates that this combinatorial effect of ARGOS is caused by ethylene with its interaction with cytokinin. The transcriptional outcome of the interaction of ethylene and cytokinin is the same in etiolated and light-grown seedlings.

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4. Introduction (Part B)

4.1 1-MCP Inhibits Ethylene to Delay Ripening

As already described that ethylene plays an essential role in plant growth and development. But continuous production of ethylene leads to plant senescence and leaf abscission. It is also important for the ripening of fruits but continuous production also leads to the over-ripening of fruits (Iqbal et al., 2017). 1-MCP acts as the inhibitor of ethylene by blocking the ethylene receptors. Its binding to the ethylene receptors reverses the ripening and senescence effects of ethylene in plants. It is a competitive inhibitor of ethylene and binds to the receptors more efficiently in the presence of ethylene. Inhibition of ethylene production in fruits by 1-MCP is done by reducing the levels of ACO and ACS genes (Li et al., 2020). Ripening of fruits is mainly carried out by ethylene. There are two types of fruits depending on their ripening patterns and are divided as climacteric and non-climacteric. Climacteric fruits are those that can ripen post-harvest and non-climacteric fruits cannot ripen post-harvest and ripen on plants. (Fukano and Tachiki, 2021). Ethylene production in fruits is described as System 1 and System 2. In climacteric fruits, ethylene production starts during the development of fruits. At this stage, fruits show a basal level of ethylene production. This basal level of ethylene production at the onset of fruit development is said to be System 1. As the fruit continues to develop and reaches maturity a burst of ethylene occurs and rises the levels of ethylene in fruits and is said to be System 2. It is autocatalytic, it keeps on producing ethylene after harvest. Non-climacteric fruits do not show such patterns (Aizat et al., 2013). Ethylene production in System 2 is responsible for fruit ripening in climacteric fruits and is also said to be autocatalytic. Non-climacteric fruits on the hand remain in System 1 and do not exhibit System 2 (Fukano and Tachiki, 2021).

4.2 Role of Ethylene in the Agriculture Industry

Plant hormone ethylene has significant importance in agriculture as it influences the developmental and ripening processes in fruits and plants. Ethylene response in fruits and vegetables is a major commercial concern as too much ethylene can spoil production. Different methods are tested and employed to control ethylene production

during the transport and storage of fruits and vegetables. It is also intentionally applied to plants to induce flowering. Ethephon is commercially available ethylene in liquid form (Chang, 2016). Similarly, several synthetic compounds are available to slow down fruit ripening by blocking the action of ethylene, which suppresses or inhibits ethylene action. Diazocyclopentadiene and 2,5-norbonadiene have shown the ability to control both the ripening and softening of apples (Blankenship and Sisler, 1993). Other compounds which can be potentially used for this purpose are aminooxy acetic acid (AOA), aminoethoxyvinylglycine (AVG), and silver thiosulfate (STS) (Abeles et al., 2012). However, commercial acceptability of these compounds is not possible due to toxicity and industrial concerns (Sisler, 2006b). In many advanced countries, 1-methylcyclopropene (1-MCP) has been used to increase the postharvest life of many fruits (Biswas et al., 2014). 1-MCP has a competitive affinity to ethylene receptors and blocks ethylene perception by preventing ethylene binding (Sisler and Serek, 1997). MCP utilization in Apple postharvest technology is considered as a breakthrough for maintaining quality for consumers. 1-MCP sustains many physiological parameters in apples that include apple taste and sugar content, firmness, and titratable acidity (tartness). 1-MCP is usually applied as a gas for a short time (2-24 hours) at very low concentrations and being nontoxic, does not have any toxic effect on the fruit consumer (Huber et al., 2003). 1-MCP affinity for the receptors is almost ten times higher than that of ethylene, therefore it is considered as highly effective even at lower concentrations (Blankenship and Dole, 2003).

When we talk about ethylene signaling in climacteric fruits tomato has great importance. As most of the ethylene signaling is studied in tomatoes (Liu et al., 2015). Tomatoes are important because of their daily consumption in food. It is concerned that their production and storage should be enhanced so that their consumption does not exceed their production. Tomato (*Lycopersicum esculentum*) is known to be a vital fruit used worldwide. Tomato belongs to the *Solanaceae* family (Khokhar, 2013). Tomatoes are part of a balanced and healthy diet. Tomatoes can be consumed raw in salads and several dishes like soups, sauces, cooked vegetables, meat, and fish to make gravy purées, juices, and ketchup. Tomatoes are rich sources of dietary fibers, vitamins, minerals, flavonoids, carotenoids (β-carotene, xanthophyll, and lycopene), phytochemicals, and many phenolics (Frusciante et al., 2007), which help in preventing many chronic diseases including cardiovascular disease (CVD) and

cancers. Tomatoes are necessary for daily diet due to their anti-mutagenic, anti-cancerous, and antioxidant activities due to carotenoids and phytochemicals (Rao and Agarwal, 1998). Ripened tomato fruit usually has a very short shelf life and can be stored for up to 7-10 days only after picking. Pakistani farmers lack proper training and technological facilities to enhance the shelf life that in turn results in loss of tomato yield due to spoilage by over-ripening (Khokhar, 2013). To prolong the shelf life of tomatoes, several handling, storage, and transport practices are being used (Mursalat et al., 2013). Pakistan has to import tomatoes from Afghanistan, Iran, and India because the locally produced tomatoes are not enough due to a lack of storage facilities. Tomatoes from Baluchistan do not fully ripen due to cold weather, and tomatoes from Sindh cannot be usually consumed due to unexpected delays/damages during transportation to other parts of the country during warm weather without temperature-controlled containers. The federal government thus decided to import tomatoes from other countries to overcome the shortage (Rizvi, 2017). Although post-harvest experts have controlled this issue and have increased the storage time of fruits and vegetables by controlling humidity and temperature conditions to keep the fruits fresh in a controlled atmosphere in advanced countries (Huber et al., 2003), Pakistan lacks the advanced transportation facilities that result in loss of tomato yield due to spoilage and over-ripening.

4.3 Physiological Parameters of Fruit Ripening:

The ripening process in fruits has a great impact on physiology along with environmental factors. The whole process of ripening involves changes in color, texture, aroma, and most importantly the taste of the fruit. All these physiological parameters contribute to the appearance of the fruit which is very important for the customer when it comes to buying (Chen et al., 2020).

Two qualities of the tomato are color and firmness that makes it attractive for a customer to buy. Firmness of the tomato is a major issue as it is difficult to maintain in storage conditions. And tomatoes being climacteric fruits continue to ripen which affects the quality of firmness. Softening of tomatoes causes a decrease in their shelf life (Batu, 2004). The color and firmness of the fruit depend on the carotenoids and polyphenolic compounds. They are found in tomatoes in large amounts. They not only attribute to the physical appearance but also add flavor and aroma to the fruit (Tohge

and Fernie, 2015). The ripening process leads to changes in the biochemistry and physiology of the fruit. As it proceeds, the chloroplast that causes photosynthesis

changes to chromoplast giving it a heterotrophic property. It is responsible for color change during the process. Large amounts of carotenoids and lycopene dominate as it proceeds. These changes make the fruit palatable and add aroma (Carrari and Fernie, 2006). The ripening process initiates pathways that lead to the formation of sugars, pigments, polyphenolic compounds, and volatiles. They add color, texture, aroma, and taste to the fruit to make it attractive and also cause softening of the fruit for easy dispersal of the seeds (Matas et al., 2009).

4.4 Control of Fruit Ripening by Gene Regulators:

It was believed for a long time that ethylene is the only major hormone that controls the ripening process in fruits. But other very important gene regulators control the ripening process with ethylene. RIN, a transcription factor regulates the expression of many ripening genes of ethylene signaling pathway *ACS, ACO, ERFs*, and other genes like *CNR, NOR,* and *PSY*. Fruits produced by rin, nor and cnr mutants had inhibited ripening, which could not be restored by the application of exogenous ethylene. Thus deciphering the importance of the gene regulators in the ripening process along with ethylene (Liu et al., 2015). Ethylene biosynthesis can be regulated by the RIN by interacting with ASC and ACO promoters. The expression of RIN can also be controlled by the feedback mechanism of ethylene. RIN and ethylene control the ripening and they regulate the expression of each other (Liu et al., 2015). Production of low endogenous ethylene in RIN-deficient tomatoes can initiate ripening such as carotenoid synthesis. But the process of full ripening that occurs after the rise of ethylene in System 2 requires the RIN. This shows that RIN is not required for the initiation of ripening which is done by the ethylene but it is required in System 2 (Li et al., 2020).

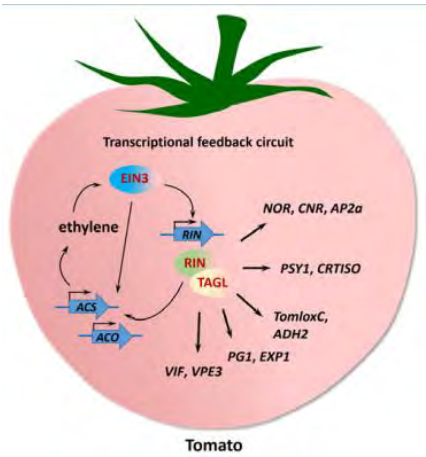

Figure 4.1: Gene Regulation pattern of ripening-related genes in tomato (Chen et al., 2020). Ethylene regulates the expression of several ripening-related genes and is regulated in a feedback mechanism by RIN.

Epigenetic regulation has an important contribution to the ripening process. Hypermethylation and demethylation of the cytosines present in the promoter region of the genes decide the activation or inactivation of ripening-related genes. Colorless Non-ripening (CNR) gene encodes a transcription factor that controls the expression ripening. This gene is said to be a part of the epigenetic trigger of ripening in tomatoes (Eriksson et al., 2004). CNR expression decrease is associated with a colorless pericarp of fruit and hence taste and texture will also be affected if it is silenced completely (Fujisawa and Ito, 2013). *Cnr* mutant which refers to the epimutation occurs due to hypermethylation of cytosines in the promoter region of genes encoding SQUAMOSA promoter binding protein CNR.

Phytoene synthase 1 (*Psy1*) is the activating gene of the lycopene biosynthetic pathway. Lycopene is the enzyme that brings the bright red color to ripened fresh tomato fruit. It converts geranylgeranyl diphosphate (GGPP) to phytoene which is then converted to lycopene and the red color of the fruit is produced (Giorio et al., 2008). The change in color during the developmental stages of tomatoes is controlled by chlorophyll degradation and a high content of carotenoids, one of which is lycopene.

The enzyme responsible for the synthesis of carotenoids in tomatoes is PSY1 (Fraser et al., 2007).

4.5 Objective of the Study Part B:

To evaluate the effect of 1-MCP on physiological parameters and molecular analysis of ethylene response genes in tomatoes. To find the role of 1-MCP in delaying the process of ripening and increasing the shelf life of tomatoes.

5. Materials and Methods

To elucidate the benefits of 1-MCP in agriculture, unripe tomatoes were treated with 10uL/L 1-MCP. Different physiological parameters were analyzed to find the effect of 1-MCP on the ripening delay and prolonged storage of tomatoes. qRT PCR of several ripening-related genes was also checked to find its effects on delayed ripening.

5.1 Experimental Design

Fresh unripe, green tomatoes of 60-155 g of weight were harvested. Tomatoes were kept in three biological replicates. 10 uL/L of 1-MCP treatment was given in a closed container through a rubber septum for 24 hours and 8 days. Control tomatoes were also kept in closed containers in three biological replicates for 24 hours and 8 days. 1-MCP with 3.3% active ingredient was obtained from Qing Dao Lu Nuo Biotechnology Co., Ltd., China. After 24 hours and 8 days of treatment of 1-MCP, tomatoes were kept in storage to check the durability of tomatoes due to 1-MCP.

5.2 Preparation of 1-MCP

1-MCP was obtained from Qing Dao Lu Nuo BioTechnology Co., Ltd., China. 1.68g of 1-MCP was used to make the stock of 1000ppm 1-MCP. A small beaker was placed in a glass jar with 1.68g of 1-MCP. A small magnetic stirrer was added to the beaker to dissolve 1-MCP. Glass jar was closed tightly with a lid containing a rubber septum. A thick greasing solution was applied around the lid to block the leakage of 1-MCP. 5 ml of water was added to the beaker through rubber septum with the syringe to dissolve 1-MCP. Glass jar was then kept in a shaker to thoroughly mix 1-MCP for 1 hour.

5.3 Measurement of Physiological Parameters in Tomatoes

Physiological parameters such as color change, firmness, and weight of tomatoes were measured daily during the storage period after treatments. The weight of tomatoes was measured on a measuring scale in grams. The color change was recorded by a group of 20 experts of 20 to 40 years of age. The finger-feel method was used to record the firmness of tomatoes (Batu, 2004). Tomatoes were kept at 25° C for storage after the treatment.

5.4 RNA Extraction and Real-Time PCR of 1-MCP Treated Tomato Samples

5.4.1 RNA Extraction

RNA was extracted from both the fleshy part of tomatoes and the skin of tomatoes. TRIZOL method was used for the extraction of RNA from tomatoes. 1g of tomato pulp and skin of both control and 1-MCP treated samples were kept in liquid nitrogen and ground in a pre-chilled pestle mortar. 2ml of TRIZOL reagent was added to the ground samples. Samples were then allowed to thaw. At room temperature, samples were shifted to 2ml Eppendorf, and 200ul of chloroform was added. Tubes were vortex for 15 seconds followed by a 15-minute centrifugation at 12,000 rpm at room temperature. The supernatant was taken in another Eppendorf and 700ul of chilled isopropanol was added. Samples were kept at room temperature for 10 minutes to allow the RNA to precipitate. Then centrifugation was performed at 12,000 rpm for 10 minutes at 4^0 C. The supernatant was discarded without disturbing the pellet. Pellet was washed with 75% ethanol and centrifuged for 5 minutes at 7500rpm at 4° C. Washing step was repeated three times. RNA was eluted in 30 µl of DEPC (Diethylpyrocarbonate) water after air drying it for 15 minutes. The extracted RNA samples were run on a 1% Agarose gel to check the integrity of the RNA.

5.4.2 RNA Quantification

A nanodrop spectrophotometer (ND/-1000) was used to quantify RNA at 260 and 280 nm.

5.4.3 DNase Treatment

RNase-free DNase I enzyme (Fermentas) was added to RNA to remove any impurities. 10ul of RNA, 1µL of 10X reaction buffer with $MgCl_2$, 1µL of DNase I enzyme and 1ul of RNase inhibitor were mixed in a pcr tube. Incubation was done at 37° C for 30 min. After incubation, 1µL of 50mM EDTA was added and incubation was done at 65° C for 10 minutes. The samples proceeded for cDNA synthesis.

5.4.4 First Strand cDNA Synthesis

cDNA was made by using the commercially available Revert Aid First Strand cDNA synthesis kit (Fermentas, USA). The reagents used in the reaction were template RNA

(1µg), 100mM Oligo (dT) primer (1µl), 5X Reaction Buffer (4µl), 10mM dNTP Mix (2µl), Revert Aid M-MµlV RT (200U/L) (1µl) and Nuclease free water (Up to 20µl). The total volume of the reaction mix was 20µl.

5.4.5 Quantitative (qRT) PCR

Primers for the analysis of genes involved in inhibiting proliferation were designed with IDT (integrated DNA technologies) (Table 5.1). The annealing temperature was set at 55-60˚C for the primers used. Relative quantification of all the selected genes was done by MYGO PRO RT PCR. Three technical replicates of each selected gene were run in qRT PCR. A housekeeping gene *EF1-alpha* was used as the internal control. The difference between the target gene and *EF1-alpha* provided the fold change and relative expression of target genes. 1 ug RNA was used to make cDNA and it was diluted five times for further PCR analysis.

The reaction mixture for qRT PCR was made by adding 5ul iTaq Syber green supermix from Biorad, 1ul of primers (F+R), 1ul cDNA, and 3ul DD H_2o. 10ul of the total reaction mixture was used. 96 well plate was used to run samples in the PCR. Thermal cycling was set for 40 repeated cycles with initial denaturation set at 95°C for 10 min followed by repeated cycles with denaturation at 95°C for 15 sec, annealing temp was set at 62°C for 1min, and elongation temp was set at 72°C for 30 sec.

Table 5.1 Primers for the Analysis of Delayed Ripening in 1-MCP Experiment

Gene	Forward (5'---- 3')	Reverse (5'----- 3')
ETR1	AGAGGGAGTTGTGAGCCAGA	GCTGCAGCCCTGACTATCTC
ACS	AAAAATAATGCGGGGCTTTT	TTGTTGCTTCTTTTCCATCG
RIN	AACATCATGGCATTGTGGTG	GTGTTGATGGTGCTGCATTT
CNR	GCCAAATCAAGCAATGATGA	TCGCAACCATACAGACCATT
NOR	AGAGAACGATGCATGGTTGT	ACTGGCTCAGGAAATTATGG
PSY1	ATCTTTGGTCTTGTACCGCAA	GGCAGTTTTTGTAGGAGGCA
EF1	GCTGTCGGTGTTGTCAAGAAT	CATCACACTGCACAGTTACT

5.5 Statistical Analysis

Statistical analysis was accomplished by doing One-way ANOVA (ANalysis Of VAriance) with post-hoc Tukey HSD (Honestly Significant Difference). Comparison of different treatments within genotypes was done by Bonferroni and Holm multiple Test Calculator. (* $p < 0.05$; ** $p < 0.01$), statistics were applied to experimental samples and the wild-type controls in each case.

6. Results and Discussion

1-MCP is used for agricultural purposes to inhibit the effects of ethylene. It inhibits the ripening of fruits and vegetables (Lata et al., 2017). Ripening of tomatoes is a big issue in their storage and transportation. In Pakistan, tomatoes are consumed daily at a high rate and their demands do not match the production rate of tomatoes. Therefore, it becomes compulsory to import tomatoes from other countries (Khokhar and HRI, 2013). To avoid the import of tomatoes from other countries, the shelf life of tomatoes needs to be improved. This could be done by delaying the ripening process of tomatoes. The ripening process can be delayed by inhibiting ethylene. 1-MCP inhibits ethylene by binding to its receptors and inhibiting the expression of ethylene-regulated ripening genes (Zhang et al., 2020). 1-MCP treatment helps in the longer storage of tomatoes by delaying the ripening process. Spoilage and over-ripening of tomatoes are prevented by 1-MCP treatment in the storage of good-quality tomatoes (Taye et al., 2019). Transportation of 1-MCP-treated tomatoes to longer distances remains fresh for a longer time (Kubota and Kroggel, 2009).

To study the effect of 1-MCP on delaying fruit ripening, unripe tomatoes were treated with 10uL/L 1-MCP. 1-MCP-treated and control tomatoes were kept in storage for 16 days to check the effect of 1-MCP in delaying ripening. Different physiological parameters and molecular analysis of ripening-related genes were checked.

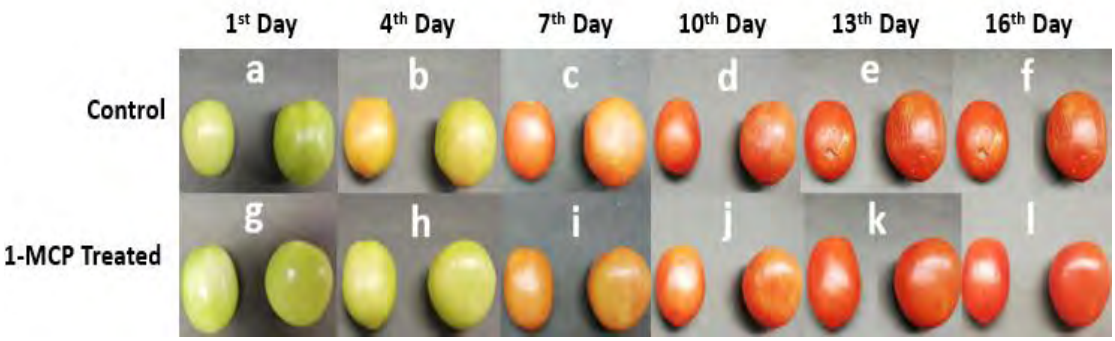

Figure 6.1: Color Change in Tomatoes for 24 hours 1-MCP Treated Tomatoes and Control Tomatoes till 16th Day of Storage. Green unripe tomatoes were kept in 10uL/L 1-MCP for 24 hours and shifted for storage at room temperature for 16 days to check the delay in ripening caused by 1-MCP by inhibiting ethylene. Control tomatoes were placed in the air.

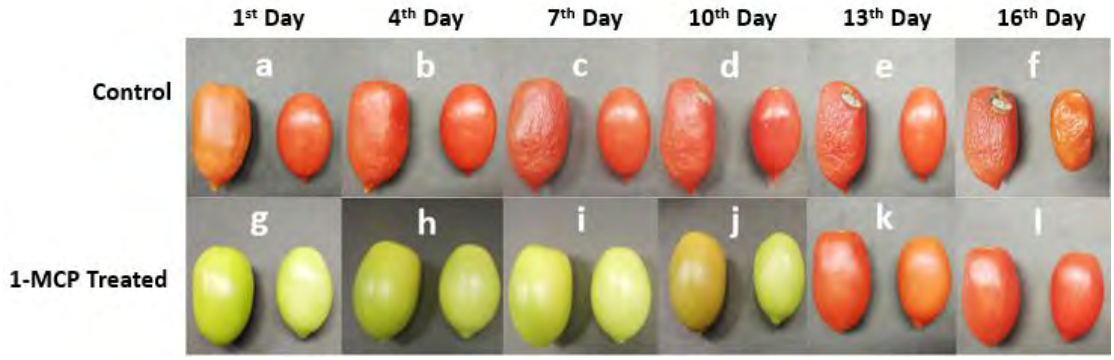

Figure 6.2: Color Change in Tomatoes for 8 days 1-MCP Treated Tomatoes until 16th Day of Storage. Green unripe tomatoes were kept in 10uL/L 1-MCP for 8 days and control tomatoes were placed in air. After 8 days of treatment, tomatoes were kept in storage for 16 days.

Table 6.1 Loss of Firmness in Tomatoes After 1-MCP Treatment

Days of Storage	Control	24 hours 1-MCP	8 Days 1-MCP
1st day	Hard	Hard	Hard
4th day	Hard	Hard	Hard
7th day	Soft	Hard	Hard
10th day	Soft	Soft	Soft
13th day	Soft	Soft	Soft
16th day	Rot	Soft	Soft

Table 6.2 Color Change in Tomatoes After 1-MCP Treatment

Days of storage	Control	24 hours 1-MCP	8 Days 1-MCP
1st day	Green	Green	Green
4th day	Orange	Green	Green
7th day	Light Red	Orange	Green
10th day	Red	Light Red	Green
13th day	Red	Red	Light Red
16th day	Red	Red	Red

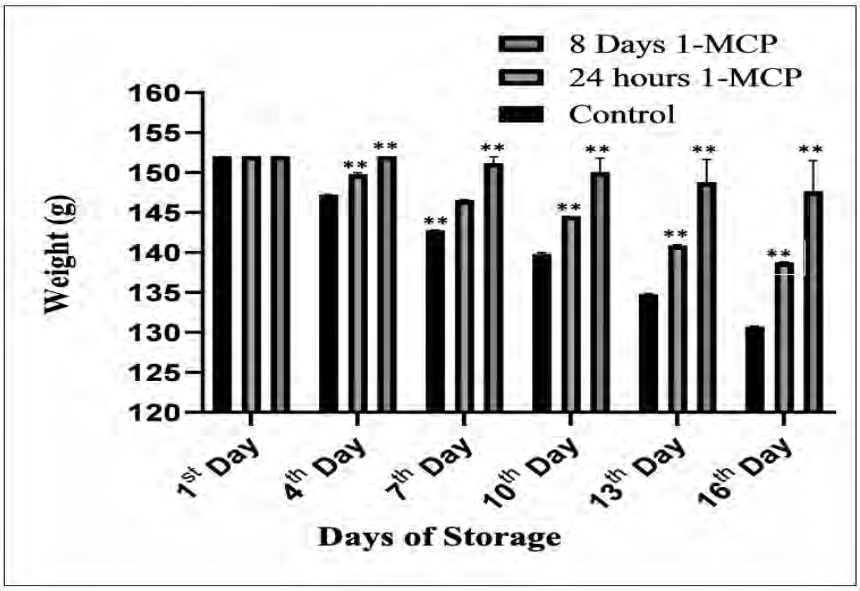

Figure 6.3: Weight Loss Data for 24-hours and 8-days of 1-MCP Treated Tomatoes in Comparison to Control till 16 Days of Storage: Columns represent the weight of tomatoes in control, 24 hours and 8-days of 1-MCP treated. Asterisks show a significant difference between control, 1-DMT, and 8-DMT (**shows $p \leq 0.01$).

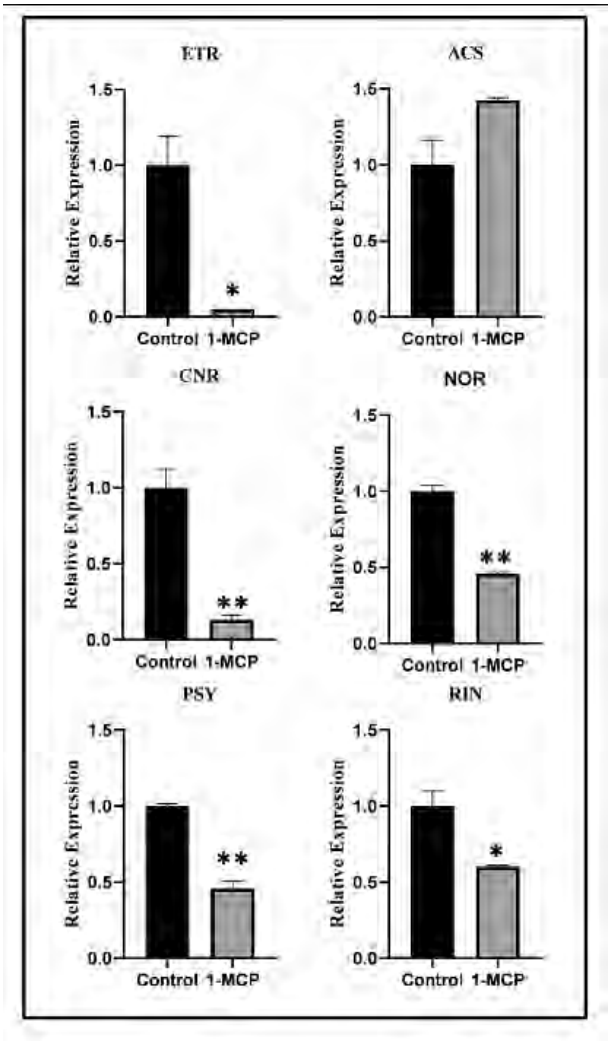

Figure 6.4: Expression of Ethylene Signaling and Ripening Genes in 24 hours 1-MCP Treated Tomatoes. Tomatoes were kept in the absence and presence of 1-MCP for 24 hours and qRT-PCR was used for gene expression. Elongation factor-alpha was used as an internal control to normalize the data and is shown as relative to the untreated control. *ETR1* and *ACS* are positive controls for ethylene. *RIN, NOR*, and *PSY1* are genes involved in the ripening of fruit. The height of each column shows the relative mRNA abundance. Error bars indicate SE using three replicates. Asterisks show a significant difference between control and 24 hours 1-MCP (*shows $p \leq 0.05$, **shows $p \leq 0.01$).

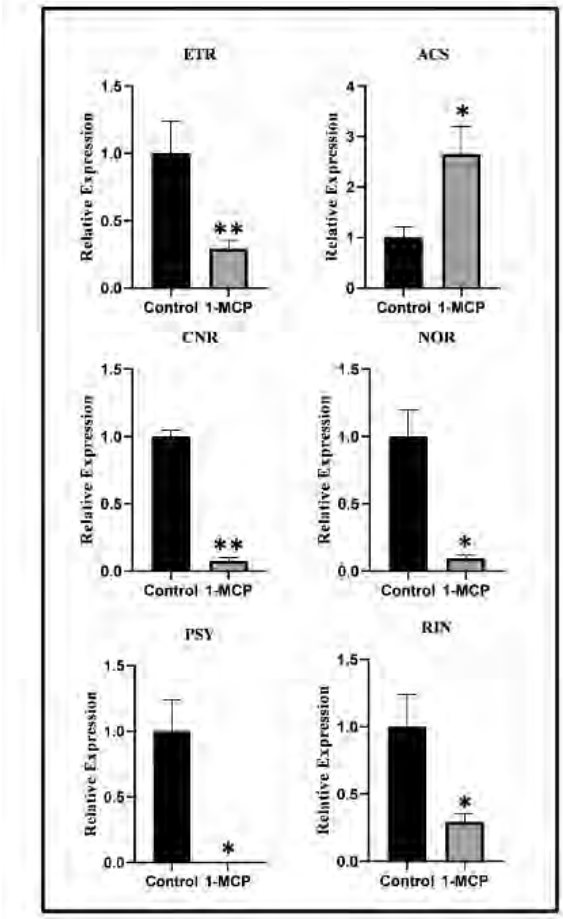

Figure 6.5: Expression of Ethylene Signaling and Ripening Genes in 8 days 1-MCP Treated Tomatoes. Tomatoes were kept in the absence and presence of 1-MCP for 8 days and qRT-PCR was used for gene expression. Elongation factor-alpha was used as an internal control to normalize the data and is shown as relative to the untreated control. *ETR1* and *ACS* are positive controls for ethylene. *RIN, NOR*, and *PSY1* are genes involved in the ripening of fruit. Y-axis shows relative mRNA abundance and error bars were added based on SE from three replicates. Asterisks show a significant difference between control and 8-Days 1-MCP (* shows $p \leq 0.05$, ** shows $p \leq 0.01$).

6.1 Physiological Parameters in Response to 1-MCP

We checked the physiological parameter and molecular expression of delayed fruit ripening in tomatoes in response to 1-MCP. We did the time course treatment of 1-MCP at two points, one for 24 hours and one for 8 days, and kept it for storage for 16 days. Two time points were used to check if longer treatment enhances the storage quality. Unripe green tomatoes treated with 10uL/L 1-MCP for 24 hours delayed color change as compared to control (Fig.6.1). Control tomatoes were softened early and were completely rotten by the 16th day of storage (Table.6.1). The 1-MCP treatment kept the tomatoes fresh and firm throughout the storage period. Slow ripening was observed in 1-MCP-treated tomatoes. Longer exposure of 1-MCP for 8 days also markedly delayed the ripening. Longer exposure retained the green color of tomatoes till the 10th day of storage (Fig.6.2). Firmness of tomatoes was also maintained throughout the storage period of tomatoes (Table.6.1). Whereas control tomatoes lost the firmness and changed the color earlier than 1-MCP treated tomatoes. Color change (Table.6.2) and firmness were better observed in longer exposure than 24 hours of 1-MCP treatment. The weight of tomatoes was also maintained throughout the storage period causing a delay in the decay of postharvest tomatoes (Fig.6.3).

In tomatoes and other climacteric fruits, a rise in the endogenous ethylene levels continues the ripening process (Taye et al., 2019). The rise in the endogenous levels that is the climacteric peak causes firmness, color change, and increased expression of genes responsible for ripening (Tichit et al., 1997). There is great post-harvest loss of tomatoes due to their low shelf life. Storage of tomatoes at low temperatures is not good enough to control post-harvest loss (Kubota and Kroggel, 2009). To control the post-harvest loss of tomatoes, the ripening process needs to be controlled by inhibiting the levels of ethylene. The application of 1-MCP effectively improved the freshness, firmness, and quality of fruits. This has been proved by many research laboratories and published (Blankenship and Dole, 2003).

The study proves that 1-MCP causes delayed fruit ripening at physiological and molecular levels. Physiological parameters are of great importance in the daily use of fruits and vegetables. Color and firmness are two important physiological parameters that attract customers to buy fruits and vegetables (Distefano et al., 2022). The rapid

change in the color and firmness leads to the low shelf life of tomatoes. There are different developmental stages of tomatoes during the process of ripening, which is mature green, breaker, turning, pink, light red, and red (Skolik et al., 2019). The physiological changes during these developmental stages are caused by the changes in the levels of ethylene (Zhao et al., 2021). Firmness is considered to be important for the transportation of tomatoes to longer distances. Physiological parameters such as nutrient content, aging, firmness, and pigmentation are controlled by ethylene biosynthesis. 1-MCP treatment delayed softening and aging in the post-harvest mangoes by turning down the enzymes responsible for fruit softening (Li et al., 2020).

6.2 Molecular Analysis of Ripening-Related Genes

Our molecular analysis of delayed ripening with 1-MCP treatment supported the physiological results. RNA was extracted on the first day of storage from both 24 hours and 8 days 1-MCP treated samples. Gene expression analysis of *ETR, ACS, RIN, CNR, NOR,* and *PSY* was done by qRT PCR. There was a marked reduction in the gene expression of ETR in response to 1-MCP in both 24-hour and 8 days treated samples (Fig.6.4 & 6.5).

1-MCP treatment reduced the gene expression of ethylene biosynthesis and signaling genes. There was a reduction in the gene expression of ethylene receptors *ETR2, ETR4, ERS2,* and *ETR5* in response to 1-MCP treatment (Gamrasni et al., 2020). Gene expression of ethylene receptors was checked in apples. Downregulation of Md-ETR 1 and *Md-ERS1* was observed in response to 1-MCP (Dal Cin et al., 2006). Gene expression of *ASC* was increased in both 24 hours and 8 days 1-MCP treated samples as compared to control (Fig.6.4 & 6.5). ACS is an enzyme of the ethylene biosynthetic pathway. The ethylene signaling pathway triggers the biosynthesis of *ACS* genes (Pattyn et al., 2021). Other hormones such as auxin, cytokinin, jasmonate, brassinosteroid, and abscisic acid also control the biosynthesis of ethylene. Biosynthesis of ASC by other hormones occurs upstream of the ethylene signaling pathway (Pattyn et al., 2021). 1-MCP inhibits the ethylene signaling pathway downstream of the ethylene receptors by inhibiting the attachment of ethylene to its receptors (Street et al., 2015). An increase in the gene expression of *LeASC2* and *LeASC4* was observed in the tomatoes that were insensitive to ethylene (Yokotani et al., 2009). Increased expression of *ASC* in our results could be due to its induction by other hormones occurring upstream of the ethylene

signaling pathway. There was no decrease in the expression of *ASC* observed in response to 1-MCP in both treatments. Crosstalk between auxin, gibberellin, and other hormones causes the synthesis of ethylene. Expression of ethylene biosynthetic genes before 1-MCP treatment continues to play a role in the process of ripening (Mir et al., 2004). *ACS* expression was increased in guava, berry, and *Arabidopsis.* The increased expression was observed due to high auxin levels (Abel et al., 1995, Yoshii and Imaseki, 1982). Treatment of Indole Acetic acid induced the expression of *ACS* genes in light-grown and dark-grown *Arabidopsis* seedlings. Transcript levels of *ASC* are influenced by the duration of light exposure and the development stages of plants (Harkey et al., 2019). Cytokinin induces and stabilizes different levels of *ACS*. Upregulation of ACS was observed in response to cytokinin (Zdarska et al., 2015).

CNR is an SPB box TF (Transcription factor) that control ripening genes in tomatoes (Gao et al., 2019). A decrease in the expression of the *CNR* gene is observed in both 24 hours and 8 days 1-MCP treated samples as compared to control (Fig.6.4 & 6.5). The control showed increased expression due to the process of ripening. The *CNR* gene encodes a transcription factor that controls the regulation of ripening-related genes. CNR is known to cause epigenetic changes in ripening-related genes (Eriksson et al., 2004). The colorless pericarp of the fruit is associated with the low expression of *CNR* which also affects the texture and taste of the fruit if the gene is completely silenced (Fujisawa and Ito, 2013). Another MADS BOX transcription factor RIN controls the ripening-related genes found in tomatoes. The expression of *RIN* and regulation of its target genes in tomatoes depend on ethylene (Fujisawa and Ito, 2013). NOR is also a MADS BOX transcription factor involved in the regulation of ripening genes. In our results gene expression of *RIN, NOR, CNR,* and *PSY* was reduced in response to 1-MCP (Fig.6.4 & 6.5). These results indicate that the expression of these genes depends on ethylene. Decreased expression of *CNR, RIN, NOR,* and *PSY* in response to 1-MCP show that they are controlled by ethylene directly or indirectly (Yokotani et al., 2009).

6.3 Conclusions of Part B

Treatment of 1-MCP delayed ripening in tomatoes and increased the shelf life of tomatoes. The shelf life of tomatoes is a big concern in Pakistan. 1-MCP treatment maintained the physiological parameters, weight, firmness, and color in tomatoes kept in storage for longer a period than control. 1-MCP treatment for 8 days had a more

pronounced effect on the physiological parameters of tomatoes. 1-MCP treatment for 24 hours also had a very good effect in delaying the ripening of tomatoes for 16 days in storage. We found that ripening-related genes like *RIN, NOR, CNR*, and *PSY* other than the genes of the ethylene signaling pathway are also ethylene dependent. 1-MCP treatment delayed the decay of tomatoes improving its shelf life making it possible to transport tomatoes to longer distances and store them for long periods.

7. Overall Conclusion

In part A we found that ethylene inhibits cell proliferation at the RAM. Reduced cell count in the cortex layer of root meristem was observed due to ethylene in *ctr1* and wild-type *Arabidopsis* seedlings. Ethylene-insensitive mutants did show reduced meristem in ethylene's response. 1-MCP recovered the reduced size of root meristem in the wild type. qRT analysis showed increased expression of *SHY2* and *ICK* genes in ethylene's response in wild type and *ctr1*. Ethylene inhibits RAM the same as cytokinin through SHY2. Cytokinin antagonizes with auxin by using the same point of convergence used by ethylene, which is SHY2. The interaction of ethylene and cytokinin was observed in the concurrent treatment of ethylene and cytokinin. Ethylene operates via multi-step phosphorelay to cause the interaction of EIN3 with ARRs of the cytokinin signaling pathway. Concurrent treatment of ethylene and cytokinin gave two types of responses in ethylene response genes. One is the additive effect as observed in *ARGOS* and the other is the subtractive effect. Reduced expression in response to concurrent treatment might be due to the sequestration of transcription factors or the degradation of transcription factors. For increased expression, there can be another possibility of expression of a new set of genes due to the combinatorial effect of these transcription factors. The transcriptional outcome of the interaction of ethylene and cytokinin is the same in etiolated and light-grown seedlings. In part B we also studied the role of ethylene in fruit ripening and used 1-MCP to delay the ripening process in tomatoes. 1-MCP treatment for 24 hours and 8 days maintained the physiological parameters like color, firmness, and weight of tomatoes till the 16^{th} day of storage. Ripening-related genes *ETR, RIN, NOR, PSY,* and *CNR* were reduced in response to 1-MCP in both treatments. Increased ACS expression indicates its production before 1-MCP treatment and its induction by other hormones. Reduced expression of ripening-related genes indicates that they are ethylene dependent. Treatment of 1-MCP delayed ripening in tomatoes and increased the shelf life of tomatoes.

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