Synechocystis: Low Dosage and Rapid Response to Ethylene

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I am submitting herewith a thesis written by Cidney Jean Allen entitled "Synechocystis: Low Dosage and Rapid Response to Ethylene." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Biochemistry and Cellular and Molecular Biology.

Brad Binder, Major Professor

We have read this thesis and recommend its acceptance:

Elizabeth Fozo, Barry Bruce

Accepted for the Council:

Dixie L. Thompson

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)
Synechocystis: Low Dosage and Rapid Response to Ethylene

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Abstract

Ethylene is a well-studied plant hormone, that has been shown to affect different aspects throughout the life cycle of plants. The ethylene receptors in plants resemble two component signaling models found in bacteria. Recent studies have identified a functional ethylene receptor, ethylene response 1 (Etr1), in sp. Strain PCC 6803. Etr1 is known for its two component signaling photoreceptor capabilities. The light signaling pathway from Etr1 involves two proteins that serve as response regulators (Slr1213 and Slr1214) and a small RNA, carbon stress-induced RNA1 (csiR1). It has been shown that Etr1 plays a role in how Synechocystis respond to ethylene and that this receptor affects phototaxis and biofilm formation. The focus of this thesis was to attain more details about cell surface changes, sensitivity to ethylene, and timing of these response in Synechocystis. Ethylene alters physiological changes on the cell surface. Application of ethylene also caused a rapid, but temporary, decrease in the transcript levels of Etr1, slr1213, and slr1214 and a rapid and prolonged decrease in csiR1 transcript. This data combined with prior reports indicate that ethylene affects a variety of processes in Synechocystis cells. Based on the data presented in this thesis, Synechocystis cells have a rapid response to ethylene and at very low dosages that are generally lower than what plants have been shown to respond to. Even at low dosages, ethylene is able regulate the transcription of many genes, causing alterations in both intra- and extracellular processes in Synechocystis. These changes lead to a variety of physiological changes and provide a good description of how these cells respond to ethylene.
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Chapter 1: Introduction

Ethylene Receptors in Plants

Ethylene is a gaseous plant hormone that plays different roles in the growth and development of plants. In earlier studies, it was found that ethylene effects seed germination, development, senescence, and fruit ripening [1]. These effects are due to receptors that respond to ethylene and transmit different signaling outputs. The majority of these discoveries were made using the model plant Arabidopsis thaliana. Arabidopsis contain five ethylene receptors called Ethylene Response 1 and 2 (AtETR1, AtETR2), Ethylene Response Sensor 1 and 2 (AtERS1, AtERS2) and EthyleneInsensitive 4 (AtEIN4). Each receptor contains a conserved ethylene-binding domain at the N terminus [2, 3] followed by GAF and kinase domains [4, 5]. Three out of the five receptors contain receiver domains at the C-terminus. The receptors in Arabidopsis are separated into subfamilies I and II. Each of the receptors contain three transmembrane alpha helices; however, receptors found in subfamily II contain additional amino acids at the N-terminal that could conceivably make a fourth alpha helix. The receptors are shown to constitutively signal in air, with the addition of ethylene inhibiting this signal output [6]. Therefore, it is believed that ethylene works as an inverse agonist. AtETR1 contains seven conserved residues in the ethylene binding domain that are required for ethylene binding; changes to any of these residues causes an ethylene insensitive, constitutively active receptor [1, 3, 5, 7, 8]. The overall architecture of these receptors is similar to that of bacterial two-component systems [9, 10] (Figure 2). Generally, two-component systems involve a ligand binding domain with a kinase domain on the C-terminus [11-13].

In bacteria, two-component systems signal by autophosphorylation of a histidine residue, followed by the transfer of a phosphate to an aspartate on a response regulator protein which is how most transcription takes place [10]. It has been proposed that a cyanobacterium passed down ethylene receptors and other two-component like receptors to plants when they acquired the cyanobacterial symbiont that became a chloroplast [10, 14, 15].

Higher Order Receptor Clusters

In bacterial two-component systems, a signal is perceived by a receptor(s) and then transmitted through a phosphor-relay mechanism to a response regulator [16]. Two-component receptors involved in chemotaxis exist as homodimers that combine to form trimers and creates a more convoluted signaling complex[17]. By forming these receptor clusters, chemoreceptors are able to activate adjacent receptors, resulting in signal amplification[16]. Previous data suggest a similar
signaling higher-order signaling complex may be taking place with ethylene receptors [18-22]. It is thought that this receptor clustering leads to the trans-activation of these receptors to promote signal amplification at the receptor level leading to the ability of plants to respond to ethylene at concentrations 300-fold below the Kd for ethylene binding[2, 23]. Ethylene receptors are able to non-covalently interact via their GAF domains and form higher order complexes. This higher-order clustering may be leading to the ability of one receptor to affect the signaling state of an adjacent receptor. This idea is supported by ethylene-insensitive mutants in *Arabidopsis* that are able to affect the signaling states of adjacent wildtype receptor isoforms [2, 18, 19, 22].

**Ethylene Receptor in *Synechocystis***

*Synechocystis* is a circular, unicellular, organism found in freshwater environments. In 1996 it was the first cyanobacterium to have its entire genome sequenced [24]. Its ability to naturally transform and readily integrate DNA into its genome by homologous recombination, makes this a beneficial organism to use for research. As stated above, it has been proposed that cyanobacteria may have evolitional ties to higher plants. In 1999, the first putative ethylene receptor was identified in the model cyanobacterium *Synechocystis* sp. PCC6803 (*Synechocystis*). It was found that the genome of *Synechocystis* contains a gene, *slr1212*, (called *Etr1* in this thesis) that encodes for a protein with a predicted ethylene binding domain [3, 7]. When the *Etr1* gene is disrupted, *Synechocystis* no longer binds ethylene suggesting that it is a functional ethylene binding protein [7].

Etr1 is an integral membrane protein that contains an ethylene-binding domain at its N terminus, followed by a phytochrome-like domain called a cyanochrome, and a C-terminal His kinase output domain [7, 25-27]. The putative ethylene-binding domain of Etr1 has the seven amino acids shown to be required for ethylene binding in the ETR1 ethylene receptor from *Arabidopsis* [3]. To directly determine whether Etr1 can bind ethylene, Lacey and Binder expressed the coding sequence for the first 130 amino acids of Etr1, the predicted ethylene-binding domain, in *Pichia pastoris* with a glutathione S-transferase (GST) tag. This resulted in high affinity, saturable ethylene-binding sites [28]. Those results supported the idea that the N-terminal portion of Etr1 can directly bind ethylene. Etr1 is one of several phytochrome-like proteins identified in *Synechocystis*; some of these proteins, including Etr1, are involved in the regulation of phototaxis [26, 27, 29-31].

Light is critical for any organism that relies on photosynthesis for growth. However, light intensity and quality can vary dramatically in both terrestrial and aquatic environments, which can significantly impact photosynthetic efficiency. Cyanobacteria is one of many organisms that has...
evolved to adapt to environmental changes. They have developed a multitude of advanced photoreceptors to sense and respond to the different intensity, quality, and directionality of light. Cyanobacteria contain a vast array of such photosensors, including the phytochromes [32, 33]. Plants, algae, nonphotosynthetic bacteria, and fungi have been shown to contain phytochromes, but they have not been identified in archaea or in animals [33]. Phytochromes form a large superfamily of GAF domain-containing photoreceptors that use bilins as their chromophore. Photoreceptors found in *Synechocystis* have been shown to play a role in light sensing physiological reactions such as phototaxis [28, 34-37]. Etr1 has been identified as one of the phytochrome-like photoreceptors known as cyanobacteriochromes or cyanochromes [38]. Cyanobacteriochromes bind linear tetrapyrrrole molecules covalently in the GAF domain and some have been shown to contain a second cysteine linkage that allows cyanobacteria to have blue-green photo reversible light sensitivity [32, 39]. Cyanobacteriochromes are one of the more unique photoreceptors found in these life forms and play a vast role in the evolutional changes that take place in cyanobacteria [32, 34, 40]. Cyanobacteriochromes allow cyanobacteria to perceive a wide range of wavelengths from UV to far-red to help adapt to environmental changes. [41].

Prior studies have demonstrated that in the presence of blue/green [38], violet/green [26], and UV/green [27] light, the cyanochrome domain of Etr1 displays photo reversible behavior. Additionally, *Synechocystis* exhibited positive phototaxis in response to red and green light, no phototaxis in response to blue light, and negative phototaxis in response to UV-A light [27, 28, 31]. Ethylene enhanced movement toward red and green light and caused cells to move toward blue light. However, ethylene had no effect on movement in response to UV-A light [28]. Collectively, these results support the idea that Etr1 functions as a receptor to reduce phototaxis toward light and that ethylene inhibits this negative regulation. This is similar to the inverse-agonist model for proposed for plants ethylene receptor [42].

**Etr1 Two-component Signaling**

It has been proposed that *Etr1* is autophosphorylated and activated by light and signals to the downstream response regulator *slr1213*, via phospho-relay (Figure 3) [27, 43]. The phosphorylated *slr1213* then binds to a region of the genome upstream of *slr1214*. It was initially thought to activate transcription of *slr1214* [27], but a recent discovery determined that the likely transcriptional target of *slr1213* is both *slr1214* and the non-coding RNA, *csiR1*, which lies in the intergenic region between *slr1213* and *slr1214* [43]. Slr1214 is a predicted AraC response
regulator that is required for the role of Etr1 in phototaxis as well as other physiological responses. The role of slr1213 and csiR1 in this signaling pathway are still unknown.

**Small RNA (sRNA)**

Outside of *E. coli*, very few sRNAs (small RNAs) have been identified in other species, and very little is known about their mechanisms [44, 45]. Some sRNAs have been shown to be vital regulators that allow the cell to alter its physiology to environmental changes. The ability of the cell to respond to different environmental changes requires a very complex process of signaling between sRNAs and protein regulators, and interactions between regulatory cascades. The regulation of sRNA-mediated effects occurs mainly by regulating the abundance of sRNAs via transcription and/or stability of mRNA (messenger RNA) [46, 47]. In recent studies, several sRNAs have been shown to play a role in regulating transcriptional and posttranscriptional levels in *Synechocystis* [48, 49]. Many of these were identified to be relevant under certain conditions important for photosynthetic growth such as light intensity and the availability of macronutrients, including nitrogen and inorganic carbon (i.e. csiR1 transcription has been found to be down-regulated in conditions of high inorganic carbon) [50, 51]. Although csiR1 has been shown to play a role in the ethylene signaling pathway, the true function of this sRNA is still unknown.

**Type IV pili, Biofilm, and Motility**

Biofilm formation is an important process for many microorganisms [52]. Biofilms occur when large populations of cells come together and adhere to a surface in response to an external stimulus. This stimulus is often identified as a stressful response on the cells causing biofilms to form to mollify the stress. Similar to the light reactive motility phototaxis, biofilm formation often requires both type IV pili and extracellular polysaccharide (EPS) secretion [53, 54]. Some bacteria do not require type IV pili for initial surface attachment, however they are required for the formation of biofilms suggesting that they help facilitate cell-to-cell interactions during biofilm formation [55]. EPS provide a protection barrier to the cells in response to stresses ranging from heavy metals to UV radiation [56]. Interestingly, it has been observed that certain sugars associated with EPS can also have a negative effect on biofilm formation [57], alluding to a complicated mechanism of signals that dictate both the type and amount of EPS produced.

Several studies have explored the nature of biofilm formation and phototaxis in *Synechocystis*. Although much has been studied regarding the role of type IV pili in motility, little work has explored the role of *Synechocystis* type IV pili in regard to biofilm formation. Fisher et al. [58]
identified two genes, *slr0977* and *slr0982*, with sequence similarity to *E. coli* genes involved in biofilm formation and surface adherence. Disruption of these genes caused alterations in EPS production and biofilm formation. Several other studies have also identified other genes that play a role in EPS secretion and biofilm formation. Both type IV pili and EPS production play a major role in mediating biofilm formation and phototaxis in *Synechocystis*. Lacey and Binder previously discovered that Etr1 signaling affects type IV pili and EPS to alter phototaxis and perhaps other physiological responses [28]. EPS contains several sugar moieties that help the bacterium adapt to environmental changes [59] and has been proven to play a role in phototaxis, spontaneous cell sedimentation, and biofilm formation in *Synechocystis* [60-62].

It has been shown that Etr1 plays a role in how *Synechocystis* respond to ethylene and that this receptor affects phototaxis and biofilm formation. The focus of this thesis was to gather more information about ethylene response in *Synechocystis* including getting more details about cell surface changes, sensitivity to ethylene, and timing of these response.

Parts of this thesis have been published in [63] or been submitted to *Frontiers in Plant Sciences* in a special issue about ethylene.
Chapter 2: Materials and Methods

Strains and Growth Conditions

*Synechocystis* sp. Strain PCC 6803 cells were acquired from the Pasteur Institute and grown on 1% (w/v)s agar in BG-11 [64] media in continuous light at 30 uM/m²/s at room temperature.

Long Term Phototaxis Conditions

Colonies of cells were placed onto 1% BG-11 agar plates, placed in containers with continuous flow of either air or air with 0.008 ppm, 0.07 ppm, 0.29 ppm, 0.7 ppm, or 1 ppm ethylene and exposed to directional white light at a fluence rate of 30 µM/m²/s for several days (as indicated in each figure) at 28°C [28]. In some experiment, RNA was isolated, treated with DNase, and cDNA synthesized in preparation for qRT-PCR (see below). Each experiment was repeated at least 3 times.

Short Term Phototaxis Conditions

For these experiments, samples were incubated in sealed chambers for up to 4 hours and exposed to directional light at 30 µM/m²/s in the presence of either air or varying concentration of ethylene from 1 ppb to 1 ppm. Instead of continuous flow chambers, ethylene at the indicated concentration was injected into the chamber through a rubber septum. For time-course experiments, samples were collected at 0, 30, 60, 120, and 240 min. after addition of 1 ppm ethylene. An air control at 240 min. was also included. Samples were collected and prepared for qRT-PCR (see below). Each experiment was repeated at least 3 times.

RNA Isolation from *Synechocystis*, DNase Treatment, and cDNA Synthesis

All RNA isolations were performed following phototaxis conditions. Following this treatment, cells were removed from the plate and resuspended in 1 mL of fresh BG-11 at equal densities. Then cells were harvested at 10,000 RPMs for 10 minutes and resuspended in 1 mL cold Trizol. Next, the cells were incubated at 95°C for 5 minutes followed by 5 minutes on ice. 200uL of chloroform was added and the cells were vortexed for 30 seconds. Cells were then incubated for 5 minutes at room temp, then cells were centrifuged for 15 minutes at 12000 x g at 4 °C. The aqueous phase (~450uL clear upper portion) was then transferred to a new tube and RNA was precipitated by addition of an equal volume (~450uL) of isopropanol. After incubating for 5 minutes at room
temperature, the precipitated RNA was pelleted by centrifugation (12000 x g) for 10 minutes at 4°C. The RNA pellet was then washed twice by in 75% ethanol with subsequent centrifugation (8000 x g) for 5 minutes at 4°C. The washed RNA was then air-dried for 5 minutes at room temp and was subsequently resuspended in 50-100μL DEPC-treated water. The concentration of RNA was then measured using a Nanodrop spectrophotometer. 8μg of the total RNA was then treated with DNase for 45 minutes at 37°C using the TURBO DNA free kit from Invitrogen in a 50μL reaction. Following inactivation of the DNase by use of the DNase Inactivation Reagent supplied with the kit, the RNA was the then further washed using the Spectrum Plant Total RNA Kit from Sigma and eluted in 50μl of DEPC-treated water. For cDNA synthesis, 800 ng of total RNA was reverse transcribed using the TaqMan Reverse Transcription Reagents Kit from Applied Biosystems in a total volume of 40μl with reagents at concentrations suggested by the manufacturer with random hexamers being used for priming. Following synthesis, the cDNA was diluted at a ratio of 1:4 for a total final volume of 160μL.

**Quantitative RT–PCR**

Following cDNA synthesis, qRT-PCR was performed using the Bio-Rad iQ5 Real-Time PCR Detection System. Each reaction was a total of 10μL with 5μL of Ssofast EvaGreen Supermix from Bio-Rad, 4μL of 1:4 diluted cDNA (1:10 final dilution), and 1μL of a 10μM forward and reverse primer mix (0.5μM each final primer concentration). Cycle times were optimized to the primers used. Transcript amounts for each cell under each condition were normalized to levels of the housekeeping gene, trpA [65]. Each gene was analyzed with two biological replicates and three technical replicates per biological replicate. Primers used are listed in Table 1. The transcript abundance for each biological replicate was then normalized to the levels of that gene in wildtype samples in air and the average ± SEM shown for all experiments.

**Lectin-binding Assay**

Isolated cells from colonies of phototaxing cells in the presence or absence of air or 1ppm ethylene for several days were treated with fluorescein isothiocyanate, FITC-conjugated lectins obtained from Sigma-Aldrich according to the methods of Wassim and others [66]. Lectins used were: concanavalin A (ConA) from *Canavalia ensiformis* to detect α-D-mannose and α-D-glucose, peanut agglutinin (PNA) from *Arachis hypogaea* to detect galactose(β1-3) N-acetyl-D-
galactosamine, and *Ulex europaeus* agglutinin (UEA) to bind α-L-fucose and N, N'-
diacetylcchitobiose. Aliquots were placed on a sterile 1% (w/v) agarose pad and observed with an
Axio Observer Z1 microscope (Zeiss) using a 100x objective and equipped with a GFP
fluorescence filter. Images of representative fields of view of cells were taken of at least three
colonies in each condition using identical exposure settings. The levels of fluorescence in 9 to 50
cells from each colony in each condition were quantified using ImageJ and the average
fluorescence intensity was determined for each replicate. For background measurements,
unlabeled cells were examined under identical exposure settings. Data were normalized to the
average background fluorescence intensity and represent the average ± SD.

**Biofilm Assay**

In some experiments we analyzed the formation of biofilm. *Synechocystis* was harvested from 1%
(w/v) agar BG-11 agar plates and resuspended in liquid BG-11 to a density of OD₇₅₀ = 0.5. Assays
were performed as described previously by [67]. 15mL of liquid culture was added to 250mL flask.
Each sample was placed in a sealed container with varying circulating ethylene concentrations
(0.008 ppm, 0.07 ppm, 0.29 ppm, 0.7 ppm) or air. Samples were incubated for 5 days with white
light at 30μM/m²/s. Non-adherent cells were removed by aspiration of medium. Then, 0.5% (w/v)
crystal violet was added for 2 min to stain cells that adhered. After removal of the stain, samples
were washed three times with 15 mL of 1x phosphate buffered saline. The biofilm-associated
crystal violet was resuspended in 10 mL of 95% ethanol for 30 min, and the OD₅₈₈ of the resulting
suspension was measured to give a measure of biofilm formation.

**Ethylene Binding Assay**

*Synechocystis* cells were grown up in 125L bioreactor. Cells were harvested and collected via
centrifugation. .8 grams of cells were spread on Whatman microfiber discs. The discs were then
folded in half and transferred to glass jars for the ethylene binding assay. Ligand competition
ethylene binding assays were performed as previously described [68].
**Table 1: Primers used for qRT-PCR analysis.**

<table>
<thead>
<tr>
<th>Primers</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Etr1</td>
<td>5’-TACAGGTGTGGGATAACGGA-3’</td>
<td>5’-CGCCACCGACATATTCATA-3’</td>
</tr>
<tr>
<td>CsiR1</td>
<td>5’-GAATGCCCCATTGGCTCAA-3’</td>
<td>5’-CCTGCAAGAACCTTGCTAAA-3’</td>
</tr>
<tr>
<td>Slr1213</td>
<td>5’-AGCCAATCATCAACAGCAAC-3’</td>
<td>5’-ACGGTAATTTCTTGTCGAG-3’</td>
</tr>
<tr>
<td>Slr1214</td>
<td>5’-AAAGTGGTTTGCATTGACGA-3’</td>
<td>5’-AAACGGCAAAGCTCATAACC-3’</td>
</tr>
<tr>
<td>Sll5043</td>
<td>5’-AGTATTTGCAAGATTATGGCCATA-3’</td>
<td>5’-CGTCCGGAGTGAATTTCCAAG-3’</td>
</tr>
<tr>
<td>Slr1452</td>
<td>5’-GAGTTAAGATGGCTGATCTT-3’</td>
<td>5’-GGTACCAATAAACAATCC-3’</td>
</tr>
<tr>
<td>TrpA</td>
<td>5’-GCGGATTTAATTGAGTTGG-3’</td>
<td>5’-GCACATCATCCAACTGACC-3’</td>
</tr>
<tr>
<td>PilB1</td>
<td>5’-CCCATCGACATGAATCTGG-3’</td>
<td>5’-TTCCTCTGGAGAGCCTTA-3’</td>
</tr>
</tbody>
</table>
Chapter 3: Results

Ethylene Alters EPS Production

It has been shown that important aspects of *Synechocystis* biology such as motility, type IV pili biosynthesis, and biofilm formation, are affected by ethylene via Etr1 [28]. Extracellular polymeric substances (EPS), which typically consist of polysaccharides [60, 69], help the bacterium adapt to environmental changes [59]. EPS has been implicated in phototaxis and biofilm formation in *Synechocystis* [58, 60, 70]. Ethylene has been shown to affect both of these physiological aspects that involves EPS, this led to the hypothesis that ethylene alters EPS production causing these physiological changes. More specifically, whether specific sugars on the cell surface were altered by ethylene treatment under these conditions. To test this, a lectin binding assay was performed using FITC-labeled peanut agglutinin (PNA) that binds to galactose(b1-3) N-acetyl-D-galactosamine, concanavalin A (ConA) that binds to a-D-mannose and a-D-glucose, or Ulex europaeus agglutinin (UEA) that binds to a-L-fucose and N, N’-diacetylchitobiose. Shown below in Figure 4, levels of background fluorescence in the absence of added lectin did not change significantly upon application of ethylene. When cells were kept in ethylene-free air, PNA, ConA, and UEA labeled the surface of cells, resulting in fluorescent intensity levels approximately 28-fold, twofold, and 1.5-fold, respectively, above the non-lectin controls (p < .05). Application of 1ppm ethylene caused an approximate twofold increase in PNA binding and a decrease in ConA binding to background levels. Ethylene caused no measurable change in UEA binding levels. These results are consistent with a model where ethylene alters the composition of EPS to affect phototaxis, and biofilm formation.

Ethylene Affects Transcript Abundance of Genes in Nearby Genomic Region

Ethylene affects different physiological aspects of *Synechocystis* via Etr1. From previous studies, a predicted model of the genes that are a part of this signaling pathway [63] has been established. The Etr1 receptor is modeled to signal to Slr1213 and Slr1214 which are encoded by genes found in the genomic region of *Etr1*. To determine how ethylene may be affecting the genomic region of *Etr1*, qRT-PCR was done to look at transcript abundance of genes found in the genomic region in the presence of ethylene. This included qRT-PCR analysis on *Slr1211*, found upstream of *Etr1* (Figure 5), *Etr1*, *Slr1213*, *Slr1214*, and *Slr1215*, which is found downstream of the signaling pathway. These cells were exposed to phototaxis conditions for 4 days. The results of this
experiment showed ethylene caused a statistically significant (p < .05) decrease in the transcript levels of Slr1215 Figure 5. The transcript levels of Slr1211 were also reduced by ethylene, but the change was below the statistical cutoff used (p = .08). This is similar to RNA-Seq data that was performed under the same conditions [63]. Although ethylene is affecting the transcript levels of these genes, ethylene did not have an effect on the genes that are part of the primary signaling pathway, Etr1, Slr1213 or Slr1214. This suggests that the genes involved in the Etr1 signaling pathway, are not affected transcriptionally by ethylene after 4 days.

Ethylene Affects csiR1 Transcriptionally

The primary signaling pathway for Etr1 also contains a recently discovered non-coding RNA known as csiR1. Recently it was discovered that the probable transcriptional target of slr1213 is both slr1214 and the sRNA csiR1, which is found in the intergenic region between slr1213 and slr1214 [43]. Since qRT results of the genomic region revealed no changes to the primary genes involved, the question arose how ethylene affects the levels of csiR1. Therefore, the transcript abundance of csiR1 was examined using qRT-PCR analysis under 4 day phototaxing conditions. The results revealed that ethylene causes approximately a threefold decrease in csiR1 transcript abundance (Figure 6). Therefore, unlike the other genes proposed to be involved in signaling from Etr1, ethylene causes a decrease in csiR1 transcript levels. The role of csiR1 has not been determined yet but this data does provide further evidence to ethylene effecting transcript levels in Synechocystis cells.

Etr1 is Required for csiR1 Transcription

The proposed signaling model for Etr1 suggest that a cascade of phosphorylation leads to the activation of downstream targets csiR1 and slr1214 [43, 63]. To determine whether the change in csiR1 transcript depends on Etr1 signaling, qRT-PCR analysis was performed to determine csiR1 transcript abundance in cells with Etr1 deleted (ΔEtr1). The csiR1 transcript levels in ΔEtr1 cells kept in ethylene-free air decreased significantly in comparison to the levels in wild-type cells, and ethylene had little or no effect on csiR1 transcript levels in this mutant (Figure 6). These results suggest that transcription of csiR1 requires Etr1 to be present to facilitate the signaling. Due to the evidence provided by Tabor et. al. that a phosphorylated slr1213 activates csiR1, this supports the predicted signaling model that slr1213 may be facilitating this interaction. More research is needed to support this.
Slr1214 Plays a Role in the Transcription of csiR1

The current models for Slr1214 function propose that it acts downstream of Slr1213 and csiR1 and is the possible output for the Etr1 signaling pathway [26-28, 43]. However, via a yeast-two hybrid assay, the Slr1214 protein could potentially have a physical interaction with the Etr1 receptor [71]. Thus, Slr1214 may act as an alternative signaling pathway or provide feedback on Etr1. To get a better idea on what may be taking place, csiR1 transcript levels were examined in cells with Slr1214 deleted (ΔSlr1214). It was previously determined that ethylene fails to alter the phototaxis of ΔSlr1214 cells [28]. Interestingly, csiR1 transcript levels increased over fivefold in air in ΔSlr1214 cells when compared to wildtype cells (Figure 6). Application of ethylene caused a decrease in csiR1 transcript levels in ΔSlr1214 cells, suggesting that Slr1214 is not required for the reduction in csiR1 transcript levels by ethylene. This data is consistent with the model where csiR1 is upstream of Slr1214 and slr1214 functions in negative feedback on this pathway. However, it is also possible that the deletion of Slr1214 had other effects such as simply stabilizing csiR1 transcript resulting in higher levels.

Synechocystis Cells Respond to Ethylene within 4hours

Previous studies done in the Binder lab have shown ethylene effecting the physiological and transcriptional characteristics of Synechocystis cells after a 4 day treatment [28, 63, 72] However, kinetic studies done on Arabidopsis have shown that seedlings are able to respond to ethylene within 15minutes of treatment [23]. Therefore, the idea arose to examine the transcript levels of genes involved in Etr1 signaling, within the first 4hr of ethylene exposure to determine just how fast these cells may be responding to ethylene. To do this, cells were maintained in phototaxis conditions for 1 day in ethylene-free air and then were treated with 1ppm ethylene for 0, 30, 60, 120, or 240 min. Air controls at 240 min were also examined. Figure 7 shows that Etr1 has a small and short-term decrease at 120 min and Slr1213 may have a small biphasic response where it first increases and then transiently decreases. Thus, both of these genes have small, rapid changes in response to the application of ethylene that are transient but by 4 days, there is no apparent effect of ethylene on these gene transcripts. By contrast, Slr1214 transcript levels decreased over 10-fold after 30 min of ethylene treatment and remained low by 240 min. This suggests that the return of Slr1214 transcript to pretreatment levels by 4 days is slower than for Etr1 and Slr1213. Similar to
what is presented in Figure 6, *csiR1* levels decreased within 30 minutes of ethylene treatment and continued to decrease as time went on.

**Synechocystis Cells Respond to Low Dosages of Ethylene**

The ability to respond to very low levels of ethylene also occurs in ethylene receptors in higher plants [23]. Therefore, we wanted to see at what concentration of ethylene cause physiological changes. To determine this, we first looked at biofilm formation of WT liquid cultures that were exposed to different concentrations of ethylene for four days in diffuse light. Using crystal violet staining, we noticed an increase in biofilm formation as ethylene concentration was increased to 0.29 ppm ethylene (Figure 8). At 0.7 ppm ethylene, cell adherence was reduced back to levels similar to air. Because we saw such small increases in biofilm, we decided to also examine changes in the transcript levels of the genes in Etr1 signaling pathway. To determine this, we injected smaller concentrations of ethylene from 0.001 ppm to 1 ppm, with cells in phototaxis conditions. Because we were not able to create a flow through method for this treatment, cells were only incubated for up to 4 hrs in a sealed chamber where ethylene was injected. Figure 9 shows *Etr1* and *Slr1213* not being affected by the lower concentrations. Similar to what we saw before, *csiR1* transcript levels decreased with concentrations as low as 0.001 ppm. These results suggest that lower concentrations of ethylene affect *Synechocystis* cells physiologically and transcriptionally. Within the 4 hr treatment of ethylene, *Slr1214* transcript levels increase first which represents an initial response to ethylene at a very low dosage. As the dosage increases, the transcript levels start to decrease but will slowly start to increase as time goes on and will eventually return to the transcript levels shown in Figure 5. These results for *csiR1* and *Slr1214* suggest a dose dependent saturation that could be altering the transcript levels (or signaling). When *Synechocystis* cells interact with ethylene, the initial small dose response increases transcription of *Slr1214*, but as the cells become more saturated, the transcript levels reach a threshold and return to levels seen in air.

**Synechocystis Cells Bind Ethylene Amounts as low as .01 ppm**

To determine how the binding affinity correlates with the physiological and transcriptional changes that have been shown in this thesis, an ethylene binding assay was performed using lower concentrations of ethylene. The results shown in Figure 10 suggest that *Synechocystis* cells can detect ethylene in dosages as low as .01 ppm. This is similar to binding activity shown in plants.
This data suggests \textit{Etr1} in \textit{Synechocystis} cells have a similar binding affinity to the ethylene receptors found in \textit{Arabidopsis}. This also correlates with the idea that ethylene receptors form trimeric dimer clusters that allow small amounts of binding to trigger a larger signaling output.
Chapter 4: Discussion

Ethylene receptors in non-plant species have not been studied as thoroughly as the ethylene receptors in plants. Many putative ethylene receptors have been identified in non-plant species but only one organism, *Synechocystis*, has been proven to actually contain a functional ethylene receptor [7, 28]. Previous studies exploring the ethylene receptor found in the cyanobacterium *Synechocystis* have shown how this receptor affects physiological and biochemical processes while functioning as a photoreceptor as well [26-28, 38, 43]. These studies have helped to determine the signaling pathway for Etr1. Etr1 contains a His kinase domain that autophosphorylates and signals to a downstream target, Slr1213. This signaling continues downstream to two additional targets, *csiR1* and *slr1214*. The information gathered from previous work led me to wonder if these effects were dose- or time-dependent.

The model proposed by Lacey and Binder suggest that one effect of ethylene is to alter the extracellular surface of the cell. Lacey and Binder recently showed that Etr1 signaling affects extracellular components such as type IV pili and EPS [28], and it is known that both type IV pili and EPS affect phototaxis, biofilm formation, and spontaneous cell sedimentation of *Synechocystis* cells [58, 60, 70, 73-75]. We wished to know whether the changes in gene transcripts were reflected in biochemical changes. The lectin binding assay showed that ethylene does not simply cause a global increase or decrease in specific sugars of the EPS. Rather, ethylene is having different effects on specific carbohydrates on the cell surface since ethylene caused a decrease in ConA binding and an increase in PNA binding, indicating a decrease in either α-D-mannose or α-D-glucose or both and an increase in galactose (β1-3) N-acetyl-D-galactosamine (Figure 4). These results along with the discovery of several other genes linked to EPS formation being affected by ethylene treatment [63], provide support for a model where ethylene signaling in *Synechocystis* alters the physiology of the cells by regulating extracellular components.

The time-course of changes in the protein coding genes near *Etr1* on the genome was also examined. This shows ethylene may be causing a small rapid and transient change in *Etr1* and *slr1213*. More obvious was that ethylene caused a rapid decrease in *slr1214*. However, after 4 days of ethylene treatment, *slr1214* transcript returned to air-control levels indicating that the effects on this gene are transient. These results indicate that these cells are responding to ethylene
and more importantly two of the genes that play a role in this signaling pathway are being affected by ethylene within minutes.

It has been suggested that \textit{csiR1} and \textit{slr1214} transcripts should have similar patterns of change because they are hypothesized to be co-transcribed from a common start site for transcription, [43, 50, 51, 76]. However, in contrast to \textit{slr1214}, application of ethylene caused a rapid decrease in \textit{csiR1} transcript levels which remained low even after 4 days of treatment. The fact that \textit{csiR1} levels remained low while \textit{slr1214} levels returned to pre-treatment levels suggests that these two genes are controlled independently by ethylene. Additionally, the deletion of \textit{Etr1} led to a reduction of gene transcript for \textit{csiR1} in both air and ethylene treatments (Figure 6). Furthermore, when \textit{slr1214} is deleted, \textit{csiR1} transcript levels are relatively high under air treatment as opposed to transcript levels seen in WT cells. Yet, in the presence of ethylene, a significant decrease still takes place. This data supports the idea that \textit{Etr1} is required for \textit{csiR1} transcription and that \textit{slr1214} may play a role in regulating this signaling complex.

Plants respond to low levels of ethylene [77]. To determine if \textit{Synechocystis} also responds to low levels, we examined the dose-responses of these cells to ethylene and found that they can physiologically respond to as low as 0.07 ppm. A lower threshold of 0.01 ppm was observed for ethylene-induced changes in gene transcript levels. To determine if these changes correlate with ethylene binding to the receptors, ethylene binding assays were performed. The results suggest that \textit{Synechocystis} cells can bind ethylene down to 0.01 ppm which is similar to what has been observed in plants [2]. These data also support the idea that \textit{Etr1} could be forming higher order receptor clusters that allows low binding to be amplified to enhance signaling output.

Based on the data presented in this thesis, \textit{Synechocystis} cells respond rapidly to ethylene and at very low dosages that are generally lower than what plants have been shown to respond to. Even at low dosages, ethylene is able regulate the transcription of many genes, causing alterations in both intra- and extracellular processes in \textit{Synechocystis}. These changes lead to a variety of physiological changes and provide a good description of how these cells respond to ethylene. This data is important for the survival of \textit{Synechocystis} and give a better understanding of how these cells respond to different concentrations of ethylene. Ethylene found in the environment can vary depending on location. Although this thesis only looked at one specific species of cyanobacteria,
it still provides valuable information to consider when studying other bacteria in regard to ethylene response. With the research of ethylene receptors expanding into other types of bacteria, the data presented in this thesis gives key variables to consider when determining which dose and time point to use to study ethylene responses.
Chapter 5: Conclusion and Future Directions

Prior to this work, many aspects of Synechocystis were determined to be affected by ethylene. Ethylene signaling via Etr1 has been shown to effect phototaxis, biofilm formation, cell sedimentation, etc. Several studies have also identified key downstream targets that play a role in this signaling pathway. Knowing that Etr1 functions as a dual receptor that responds to both light and ethylene it is important to determine how each of these cues’ effects signaling. From the model proposed by Lacey et. Al. [63], it has been determined that in the presence of light, Etr1 becomes autophosphorylated on its his-kinase domain. This triggers a downstream cascade of phosphorylation to take place in which the response regulator Slr1213 becomes activated. Slr1213 then proceeds to activate the sRNA csiR1 and another response regulator Slr1214. Slr1214 has been shown to be required for ethylene signaling output that effects several aspects including motility, EPS, and type IV pili. The data presented in this thesis further supports the signaling modal by demonstrating that ethylene alters aspects of the cell surface and effects transcription of different genes involved in the signaling pathway. Although this data supports the predicted signaling model, there are still many questions that need to be answered.

For instance, the signaling interactions between Slr1213, csiR1, and Slr1214 could be studied more to determine the signaling output. Sato et. al. has shown that Slr1214 directly interacts with Etr1 but with the recent finding of csiR1 upstream of Slr1214[43], and Tabor et. al. showing Slr1213 activating csiR1, these results could be interpreted differently. More research is needed to thoroughly understand the protein-protein interactions taking place in this signaling pathway.

The roles of the downstream targets, Slr1213, csiR1, and Slr1214 are unknown and could also provide a lot more information on what is taking place. Deleting Slr1213 and csiR1 could provide more insights on the roles of these genes in this signaling pathway. The lack of information and placement of csiR1 might pose a challenge for deletion but if successful, could shed light on its role in this pathway and possibly others.

The receptor itself could also be further characterized to understand the interaction of the ethylene binding and cyanochrome domains as well as role of the histidine kinase in this signaling pathway. Further characterization of the ethylene binding domain could help to identify the location of this receptor in Synechocystis. This could also help to determine if this receptor forms clusters similar
to the ethylene receptors found in plants. These clusters are thought to form at the GAF domain on ethylene receptors and would further support the findings of my thesis in that Etr1 is forming a trimeric dimer cluster that allows a large signaling output from very low concentrations of ethylene.
References


Appendix
Figure 1: Ethylene Receptors found in Arabidopsis. Arabidopsis contains five ethylene receptors. Each receptor contains three transmembrane helixes that make up the ethylene binding domain, followed by a GAF domain and a histidine kinase domain. AtETR1 and AtERS1 make up subfamily 1 and AtETR2, AtEIN4, and AtERS2 make up subfamily 2. Receptors in subfamily 2 contains additional amino acids on the N-terminus. Three of these receptors contain a receiver domain on the C-terminus. The general architect of these receptors is similar to two component systems found in bacteria.
Figure 2: Architectural comparison of two component system, AtETR1 found in Arabidopsis, and Etr1 found in Synechocystis. Two component systems generally consist of a ligand that binds a specific component, followed by a histidine kinase domain that initiates autophosphorylation and then passes a phosphate to an aspartate residue on the response regulator. AtETR1 and Etr1 share a similar structure. Etr1 consist of PAS/PAC domain with the GAF domain and the downstream response regulator that will receive the phosphate is a transcription factor known as slr1213.
Figure 3: Etr1 signaling with and without ethylene. In the presence of light Etr1 becomes autophosphorylated on the histidine kinase domain that leads to a cascade of phosphorylation. Slr1213 becomes phosphorylated and then activates csiR1 and Slr1214. This signaling output leads to an inhibition of phototaxis and biofilm formation. Ethylene acts as an inverse agonist in which it inhibits this signaling process and leads to an increase in phototaxis and biofilm.
Figure 4: Lectin Binding Assay. Fluorescent images of *Synechocystis* cells stained with FITC conjugated peanut agglutinin (PNA), concanavalin A (ConA) or *Ulex europaeus* agglutinin (UEA). Cells were kept in phototaxing conditions for 4d in directional light in the presence of air or 1ppm ethylene. The control consists of cells that are not stained with a lectin. Quantification of fluorescence intensity from FITC-conjugated lectins bound to cells are shown on the right. The fluorescence intensity was normalized in nonlabelled cells maintained in air and represent the average ± SD. *Statistically significant difference (p < .05) caused by application of ethylene as determined by ANOVA.
Figure 5: QRT-PCR analysis of genes found in the genomic region of Etr1. Visual representation of genomic region shown above graph. *Statistically significant difference (p < .05) caused by application of ethylene as determined by ANOVA.
Figure 6: Transcript levels of *csiR1*. Using phototaxis conditions, each strain of *Synechocystis* cells were treated with air or 1ppm ethylene in the presence of directional white light for 4d. QRT-PCR was done using primers listed in the Table 1. ∆Etr1 is a strain of *Synechocystis* that has Etr1 deleted. ∆slr1214 is a strain of *Synechocystis* that has slr1214 deleted. Expression levels were first normalized to a house keeping gene, then each gene was normalized to WT in air. Data are averages ± SD. *Statistically significant difference (p < .05) caused by application of ethylene as determined by ANOVA.
Figure 7: Transcript levels of primary genes within 4h of ethylene treatment. QRT-PCR analysis was done on *Synechocystis* cells at different time points (0 minutes, 30 minutes, 60 minutes, 120 minutes, and 240 minutes) under phototaxis conditions. Air samples were collected at the beginning (0 minutes) and end (Air 240) of the experiment. Samples collected at each time point (30, 60, 120, and 240) were treated 1 ppm ethylene via continuous flow through. Expression levels were first normalized to a housekeeping gene, then each gene was normalized to air. Data are averages ± SD. *Statistically significant difference (p < .05) caused by application of ethylene as determined by two-way ANOVA.*
Figure 8: Biofilm Assay using lower concentrations of ethylene. Liquid cultures of *Synechocystis* cells were incubated for 7 days in sealed chambers with continuous flow through of air and ethylene. Biofilm assay was carried out using [31] protocol. Quantification of the biofilm formation was performed by crystal violet staining of attached cells. Optical density was measured at OD630. Data are averages ± SD. *Statistically significant difference (p < .05) caused by application of ethylene as determined by ANOVA.
Figure 9: QRT-PCR analysis of primary genes under low concentrations of ethylene.
*Synechocystis* cells were exposed to directional light and air or ethylene for 4hs. Specific amounts of ethylene were injected into the sealed chambers. Samples were collected after 4h. Cells were harvested, RNA was extracted and reverse transcribed and qPCR was performed. Expression levels were first normalized to a house keeping gene, then each gene was normalized to WT in air. Data are averages ± SD. *Statistically significant difference (p < .05) caused by application of ethylene as determined by ANOVA.
Figure 10: Ethylene binding activity of *Synechocystis* cells. For ethylene binding assays, 0.8 grams (wet weight) of cells were spread on microfiber discs. The discs were then transferred to glass jars for the ethylene binding assays, as previously described [2].
Vita

Cidney Jean Allen was born in Detroit, Michigan on October 29, 1992 to Calvin and Vivian Allen. She is the youngest daughter and first person in the family to attain a degree of this magnitude. After high school she attended Clark Atlanta University in Atlanta, GA where she received her Bachelor of Science in Biology in 2015. Later that year she began attending the University of Tennessee, Knoxville for a degree in Biochemistry and Cellular and Molecular Biology.