Ethylene Regulates the Physiology of the Cyanobacterium *Synechocystis* sp. PCC 6803 via an Ethylene Receptor[1][OPEN]

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Ethylene is a plant hormone that plays a crucial role in the growth and development of plants. The ethylene receptors in plants are well studied, and it is generally assumed that they are found only in plants. In a search of sequenced genomes, we found that many bacterial species contain putative ethylene receptors. Plants acquired many proteins from cyanobacteria as a result of the endosymbiotic event that led to chloroplasts. We provide data that the cyanobacterium Synechocystis (*Synechocystis* sp. PCC 6803) has a functional receptor for ethylene, Synechocystis Ethylene Response1 (SynEtr1). We first show that SynEtr1 directly binds ethylene. Second, we demonstrate that application of ethylene to Synechocystis cells or disruption of the *SynEtr1* gene affects several processes, including phototaxis, type IV pilus biosynthesis, photosystem II levels, biofilm formation, and spontaneous cell sedimentation. Our data suggest a model where SynEtr1 inhibits downstream signaling and ethylene inhibits SynEtr1. This is similar to the inverse-agonist model of ethylene receptor signaling proposed for plants and suggests a conservation of structure and function that possibly originated over 1 billion years ago. Prior research showed that SynEtr1 also contains a light-responsive phytochrome-like domain. Thus, SynEtr1 is a bifunctional receptor that mediates responses to both light and ethylene. To our knowledge, this is the first demonstration of a functional ethylene receptor in a nonplant species and suggests that that the perception of ethylene is more widespread than previously thought.

Ethylene is a gaseous hormone that influences the growth and development of plants (Abeles et al., 1992). The signal transduction pathway for ethylene has been studied predominantly in the flowering plant Arabidopsis (*Arabidopsis thaliana*), but research on plant species from more ancient lineages suggests that ethylene signaling probably evolved in plants prior to the colonization of land (Rensing et al., 2008; Banks et al., 2011; Gallie, 2015; Ju et al., 2015).

In plants, the perception of ethylene is mediated by a family of receptors that contain a conserved N-terminal transmembrane ethylene-binding domain consisting of three transmembrane α-helices with seven conserved amino acids required for the binding of ethylene (Schaller and Bleecker, 1995; Wang et al., 2006). Several of these amino acids are believed to coordinate a copper cofactor required for ethylene binding (Rodríguez et al., 1999). These receptors have homology to bacterial two-component receptors that function via His autophosphorylation, followed by transfer of this phosphate to an Asp residue on a downstream response regulator protein (Chang et al., 1993). Plants acquired many proteins from cyanobacteria as a result of an endosymbiotic event approximately 1.5 billion years ago that led to chloroplasts (Yoon et al., 2004). Because of the endosymbiotic gene transfer that occurred, it has been proposed that components of several two-component-like receptors in plants, such as ethylene receptors and phytochromes, were acquired from the cyanobacterium that gave rise to the chloroplasts of plants (Kehoe and Grossman, 1996; Martin et al., 2002; Mount and Chang, 2002; Timmis et al., 2004; Schaller et al., 2011).

Phytochrome-like receptors (Vierstra and Zhang, 2011), but not ethylene receptors, have been characterized in nonplant species. Some cyanobacterial species have saturable ethylene-binding sites and contain genes predicted to encode proteins with ethylene-binding domains (Rodríguez et al., 1999; Wang et al., 2006), but the distribution and function of ethylene receptors in bacteria are unknown. In a search of sequenced genomes, we found that genes encoding putative ethylene receptors are found in diverse bacterial species. One of these genes, *slr1212*, is in the model cyanobacterium Synechocystis (*Synechocystis* sp. PCC 6803). We previously showed that disruption of this gene eliminates ethylene-binding activity in Synechocystis, leading to the speculation that it encodes an ethylene-binding protein (Rodríguez et al., 1999). This gene, called *Synechocystis Ethylene Response1* (*SynEtr1*), as originally designated by Ulijasz et al. (2009) because of its putative role as an ethylene receptor, also has been called *Positive*

[1] This work was supported by the U.S. National Science Foundation (grant no. IOS–1254423 to B.M.B.).

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Both authors conceived, designed, performed, and analyzed the experiments and wrote the article; B.M.B. supervised the research.

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www.plantphysiol.org/cgi/doi/10.1104/pp.16.00602

Phototaxis A (Narikawa et al., 2011) and *UV Intensity Response Sensor* (Song et al., 2011), because of its role in light signaling. Despite these observations, there has been no research published that demonstrates that SynEtr1 directly binds ethylene or functions as an ethylene receptor.

We focused on SynEtr1 to determine whether it is a functional ethylene receptor. Expression of the N-terminal portion of SynEtr1 in *P. pastoris* led to the generation of ethylene-binding sites, demonstrating that this region of the protein directly binds ethylene. Treatment of Synechocystis with ethylene or disruption of SynEtr1 caused measurable changes in physiology, including faster movement toward light, slower cell sedimentation, enhanced biofilm production, a larger number of type IV pili, and higher levels of PSII. Additionally, SynEtr1-deficient Synechocystis cells transformed with a mutant SynEtr1 that cannot bind ethylene do not respond to ethylene. Our research demonstrates that SynEtr1 is an ethylene receptor and, in the context of prior research (Ulijasz et al., 2009; Narikawa et al., 2011; Song et al., 2011), likely functions as a dual input receptor for both light and ethylene. To our knowledge, this is the first report of a functional ethylene receptor in a cyanobacterium, making it the first ethylene receptor characterized in a nonplant species.

RESULTS

Putative Ethylene Receptors Are Found In Many Bacterial Species

We previously documented that several nongreen plant species contain proteins with putative ethylene-binding domains (Wang et al., 2006). However, many additional genomes have been sequenced since this initial report. Therefore, we were curious to know the extent, distribution, and domain structure of ethylene receptors in nonplant species. A BLAST search excluding plant species was performed using the amino acid residues that form the ethylene-binding domain of ETR1 from Arabidopsis (amino acids 1–130) as the query sequence. This revealed that 112 bacterial species contain proteins with domains that have the seven amino acid residues that are required for ethylene binding in plant receptors and, thus, are predicted to bind ethylene (Supplemental Fig. S1; Supplemental Table S1). Most of the bacteria were either cyanobacteria or proteobacteria, with individual strains predicted to contain between one and seven ethylene receptor isoforms. Many of the proteobacteria are known to form nonpathogenic associations with plants, and none are plant pathogens. Even though all identified proteins contain the predicted ethylene-binding domain at the N terminus of the protein, there is wide variation in the domains predicted for the remainder of each protein (Supplemental Table S1). Some contain a combination of GAF (for cGMP phosphodiesterase/adenyl cyclase/FhlA), His kinase, and receiver domains much like what is seen in the ethylene receptors from plants. Others contain phytochrome-like domains and, thus, may function in the detection of both ethylene and light. Interestingly, several contain diguanylate cyclase, cyclic di-GMP phosphodiesterase, or methyl-accepting domains. This variety of domain structure indicates that these predicted ethylene receptors are likely to have diverse biochemical outputs and functions. A phylogenetic tree that was generated based on a comparison of the putative ethylene-binding domains (Supplemental Fig. S1) shows that there is a general pattern where receptors with more complex domain structures tend to cluster in the tree. Also, there are some structural similarities in closely grouped receptor homologs. However, there are no clear patterns for these similarities.

SynEtr1 Directly Binds Ethylene

One gene in this list is *SynEtr1*, which encodes SynEtr1 found in the cyanobacterium Synechocystis. This is an integral membrane protein that is predicted to contain an ethylene-binding domain at its N terminus, followed by a phytochrome-like domain known as a cyanochrome, and a C-terminal His kinase domain that is likely to be the output domain of the protein (Fig. 1A; Rodríguez et al., 1999; Ulijasz et al., 2009; Kwon et al., 2010; Narikawa et al., 2011; Song et al., 2011). The putative ethylene-binding domain of SynEtr1 has the seven amino acids shown to be required for ethylene binding in the ETR1 ethylene receptor from Arabidopsis (Fig. 1B; Wang et al., 2006). We previously found that Synechocystis has ethylene-binding sites, and disruption of the *SynEtr1* gene locus at the second transmembrane helix eliminates these ethylene-binding sites (Rodríguez et al., 1999; Wang et al., 2006). This led to the hypothesis that SynEtr1 is an ethylene-binding protein. However, this does not demonstrate that SynEtr1 is a functional ethylene receptor. Also, it is possible that this disruption affects another protein that is the ethylene-binding site. To directly determine whether SynEtr1 can bind ethylene, we expressed the coding sequence for the first 130 amino acids of SynEtr1, which is predicted to contain the ethylene-binding domain, fused to glutathione *S*-transferase (GST; SynEtr1[1-130]GST) in *Pichia pastoris*. This resulted in high affinity, saturable ethylene-binding sites (Fig. 1C). Control experiments with *P. pastoris* containing the empty vector (pPICZ) or expressing GST alone had no saturable ethylene binding. We targeted two of the seven amino acids predicted to be required for ethylene binding, Cys-78 and His-82, as well as Cys-77 for mutagenesis to Ala. All three mutations disrupted ethylene binding in yeast (Fig. 1C). A Cys-78Ser mutation also disrupted ethylene binding. Western blots using anti-GST antibodies confirmed that all transgenes were expressed (Fig. 1C). These results show that the N-terminal portion of SynEtr1 can directly bind ethylene.

Application of Ethylene Affects Phototaxis via SynEtr1

Even though SynEtr1 binds ethylene, it is unknown whether SynEtr1 functions as an ethylene receptor that allows Synechocystis cells to detect and respond to

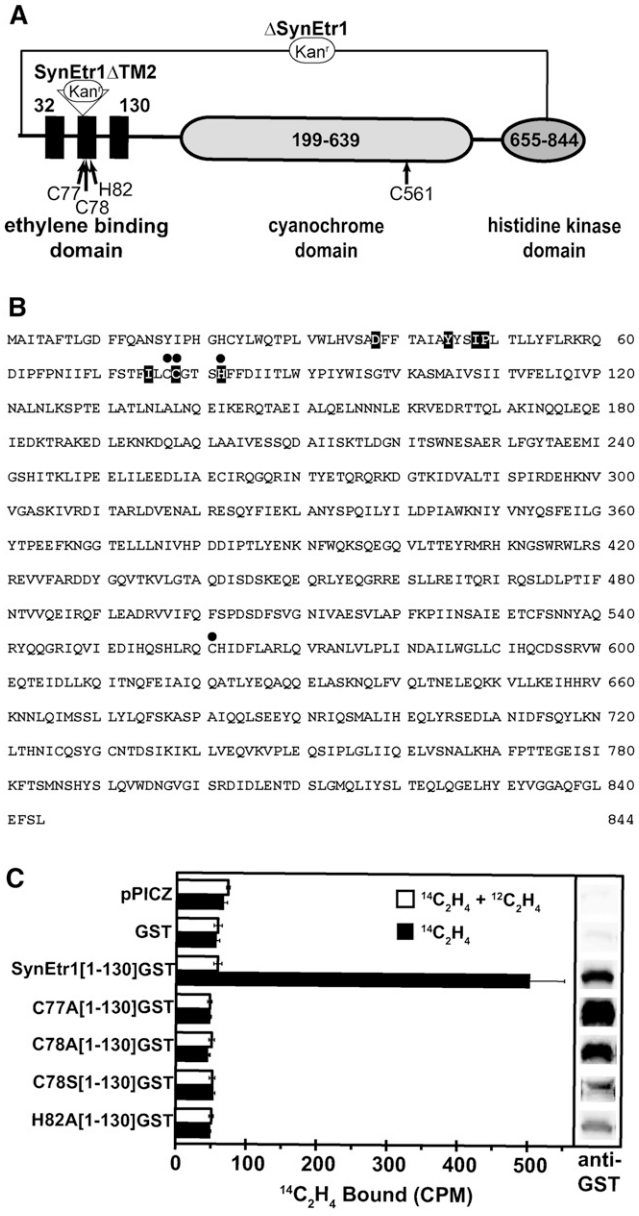

Figure 1. Ethylene binds to SynEtr1 protein expressed in yeast. A, Predicted domain structure of SynEtr1 based on sequence homology. Numbers denote the amino acid range predicted to form each domain. Black rectangles denote the three predicted transmembrane α-helices forming the ethylene-binding domain. Arrows denote the relative locations of the point mutants used. The positions of the gene disruption in the second transmembrane domain of the ethylene-binding domain (*SynEtr1*ΔTM2) and gene deletion (Δ*SynEtr1*) using a kanamycin resistance gene (Kan^r) are shown. B, Predicted amino acid sequence of SynEtr1. The seven amino acid residues that correspond to the residues required for ethylene binding in ETR1 from Arabidopsis are shaded black. Amino acids targeted for mutagenesis in this study are marked with circles. Amino acid residues are numbered at right. C, Ethylene-binding activity to equal amounts of yeast expressing the binding domain of wild-type or mutant receptors (as indicated) fused to GST, GST alone, or empty vector was compared between samples treated with [^{14}C]ethylene (0.1 μL L^{-1}) alone or in the presence of [^{14}C]ethylene (0.1 μL L^{-1}) with excess [^{12}C]ethylene (1,000 μL L^{-1}) to determine background binding activity. Data show average cpm ± SD. Proteins from membranes of yeast expressing the ethylene receptor, GST alone, or empty vector were separated by SDS-PAGE and analyzed with western blots probed with anti-GST antibodies to confirm the expression of receptors.

ethylene. SynEtr1 is one of several phytochrome-like proteins identified in Synechocystis; some of these proteins, including SynEtr1, are involved in the regulation of phototaxis (Kaneko et al., 1996; Hughes et al., 1997; Yeh et al., 1997; Park et al., 2000; Wilde et al., 2002; Narikawa et al., 2011; Song et al., 2011). We thus hypothesized that ethylene affects phototaxis. To test this hypothesis, we measured phototaxis toward directional white light (30 μmol m^{-2} s^{-1}) in the presence and absence of 1 μL L^{-1} ethylene (Fig. 2, A and B; Supplemental Figs. S2 and S3A). Directional white light caused wild-type Synechocystis cells to move toward the light. As noted in various studies, this movement often, but not always, was characterized by finger-like projections of cells moving as a group. Application of ethylene enhanced the maximum distance moved by wild-type Synechocystis toward white light ($P < 0.05$) and increased the number of cells that moved toward light. A nonmotile Synechocystis strain, *Synechocystis* sp. ATCC 27184 (Ikeuchi and Tabata, 2001; Trautmann et al., 2012), failed to move in response to directional white light in the absence or presence of ethylene, indicating that ethylene affects taxis rather than growth (data not shown). The effect of ethylene on light sensitivity also was examined by performing phototaxis assays in air and 1 μL L^{-1} ethylene at fluence rates ranging from 3.5 to 30 μmol m^{-2} s^{-1} white light. At the lowest fluence rate, no phototaxis was observed in air (Fig. 2C). Phototaxis increased with higher light levels. Ethylene enhanced phototaxis toward white light at all light intensities tested.

Prior biophysical studies have demonstrated that the cyanochrome domain of SynEtr1 has photoreversible behavior to blue/green (Ulijasz et al., 2009), violet/green (Narikawa et al., 2011), and UV/green (Song et al., 2011) light. Given this uncertainty regarding which wavelengths are perceived by SynEtr1, we measured phototaxis in response to directional monochromatic red, blue, green, and UV-A light (Fig. 2D; Supplemental Fig. S3B). In air, positive phototaxis was observed in response to red and green light, but consistent with prior studies, no phototaxis occurred in response to blue or UV-A light at the fluence rates used (Wilde et al., 2002; Song et al., 2011). Application of ethylene enhanced movement toward red and green light and caused cells to move toward blue light. By contrast, ethylene had no effect on movement in response to UV-A light.

To determine the role of SynEtr1 in this enhancement, we disrupted the *SynEtr1* gene in the coding sequence for the second transmembrane domain using a kanamycin resistance gene (SynEtr1ΔTM2; Fig. 1A). The disruption was confirmed by colony PCR (Supplemental Fig. S4), and we have shown previously that a similar disruption eliminates ethylene binding by Synechocystis

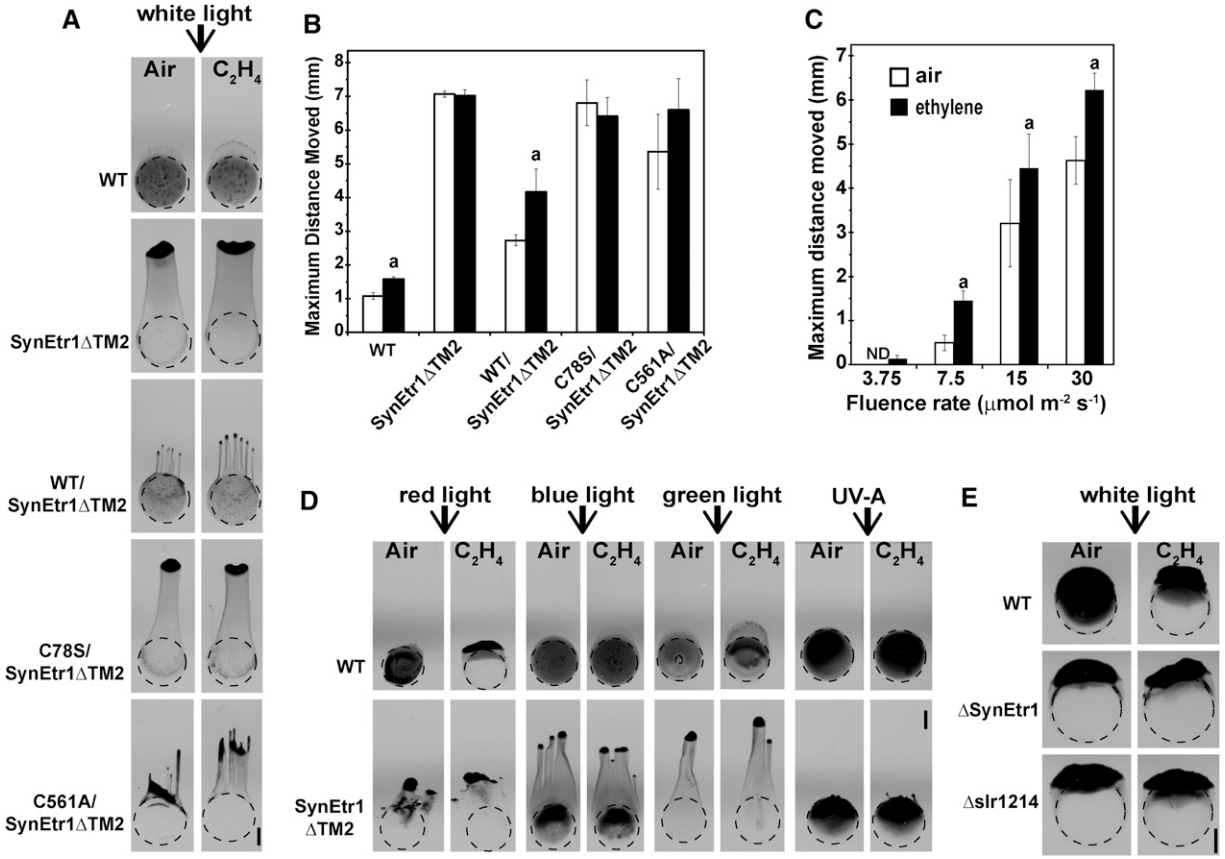

Figure 2. Ethylene and SynEtr1 affect Synechocystis motility. A, Colonies of Synechocystis strains were spotted on soft agar plates and exposed to directional white light (30 μmol m^{-2} s^{-1}) for 4 d in the absence or presence of 1 μL L^{-1} ethylene. At the end of 4 d, images were acquired of wild-type (WT), SynEtr1ΔTM2, and SynEtr1ΔTM2 colonies transformed with the full-length wild-type *SynEtr1* (WT/SynEtr1ΔTM2), *Cys-78Ser* (C78S/SynEtr1ΔTM2), or *Cys-561A* (C561A/SynEtr1ΔTM2) mutant transgene. Dotted circles mark the initial positions of spots. B, Quantification of maximum movement toward white light (30 μmol m^{-2} s^{-1}) for 4 d in the absence or presence of 1 μL L^{-1} ethylene for wild-type, SynEtr1ΔTM2, and SynEtr1ΔTM2 colonies transformed with the full-length wild-type *SynEtr1* (WT/SynEtr1ΔTM2), *Cys-78Ser* (C78S/SynEtr1ΔTM2), or *Cys-561A* (C561A/SynEtr1ΔTM2) mutant transgene. Data are averages ± SD. [a]Statistical difference caused by the application of ethylene ($P < 0.05$). C, Quantification of wild-type maximum movement toward white light after 14 d in ethylene-free air or 1 μL L^{-1} ethylene at the indicated fluence rate of light. ND denotes that movement was not detected. Data are averages ± SD. [a]Statistical difference caused by the application of ethylene ($P < 0.05$). D, Colonies of wild-type and SynEtr1ΔTM2 strains were spotted on soft agar plates and exposed to 30 μmol m^{-2} s^{-1} directional monochromatic red light for 4 d, green light for 4 d, blue light for 7 d, or 60 μmol m^{-2} s^{-1} UV-A light for 7 d in the absence or presence of 1 μL L^{-1} ethylene. E, Colonies of wild-type, ΔSynEtr1, and Δslr1214 cells were spotted on soft agar plates and exposed to directional white light (30 μmol m^{-2} s^{-1}) for 4 d in the absence or presence of 1 μL L^{-1} ethylene. Bars in A, D, and E = 2 mm.

(Rodríguez et al., 1999). SynEtr1ΔTM2 Synechocystis showed a large enhancement in the distance moved toward white light (Fig. 2, A and B) as well as red, blue, and green light (Fig. 2D). Unlike wild-type cells, SynEtr1ΔTM2 cells phototaxed toward UV-A light (Fig. 2D), which is similar to what Song et al. (2011) observed when SynEtr1 was deleted. The SynEtr1ΔTM2 cells did not respond to ethylene, supporting the idea that SynEtr1 is required for ethylene sensing. Blue light does not typically stimulate phototaxis in Synechocystis. However, disruption of another receptor, Cyanobacteria Phytochrome2, also causes Synechocystis to move toward blue light (Wilde et al., 2002). This suggests that both Cyanobacteria Phytochrome2 and SynEtr1 inhibit movement toward blue light. Generally, both application of ethylene and disruption of SynEtr1 led to enhanced phototaxis. However, disruption of SynEtr1 consistently had a larger effect on motility than application of ethylene.

A prior study on SynEtr1 deleted most of the coding sequence for *SynEtr1*, resulting in altered phototaxis in response to directional UV-A light but no change in phototaxis toward red, green, or blue light; the authors did not examine phototaxis toward white light (Song et al., 2011). Since these results are different from what we obtained with the SynEtr1ΔTM2 disruption, we generated a deletion mutant (ΔSynEtr1) similar to that of Song et al. (2011). The deletion was confirmed by colony PCR (Supplemental Fig. S4C). These ΔSynEtr1 mutants responded to directional 60 μmol m^{-2} s^{-1} UV-A light (data not shown), as observed by Song et al. (2011).

In response to directional white light (30 μmol m^{-2} s^{-1}), the ΔSynEtr1 cells showed enhanced positive phototaxis compared with wild-type cells and did not respond to ethylene, confirming that SynEtr1 is required for ethylene responses (Fig. 2E). The enhanced motility in ΔSynEtr1 was less than what was observed in SynEtr1ΔTM2.

Complementation of SynEtr1ΔTM2 with a full-length wild-type *SynEtr1* gene (WT/SynEtr1ΔTM2) resulted in a reduction in maximum phototaxis in air and a rescue of ethylene-enhanced phototaxis (Fig. 2, A and B; Supplemental Fig. S3A), confirming that ethylene affects phototaxis via SynEtr1. By contrast, complementation of SynEtr1ΔTM2 with a Cys-78Ser mutant protein that fails to bind ethylene (C78S/SynEtr1ΔTM2) failed to reduce phototaxis in air, and the cyanobacteria did not respond to ethylene with altered phototaxis. These results suggest that Cys-78 might be required for a functional protein. This is distinct from the ETR1 ethylene receptor of Arabidopsis, where the comparable mutation results in a protein that fails to bind ethylene and remains signaling (Bleecker et al., 1988; Schaller and Bleecker, 1995).

Collectively, these results indicate that SynEtr1 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 ethylene receptor signaling proposed for plants (Hall et al., 1999).

Light Signaling in SynEtr1 Is Not Needed for an Ethylene Response

Our observations coupled with prior reports (Ulijasz et al., 2009; Narikawa et al., 2011; Song et al., 2011) raise the possibility that SynEtr1 functions as a dual input receptor for both ethylene and photons. To gain further insight into the possible interplay between these two inputs, we generated a Cys-561Ala mutant and transformed this mutant into SynEtr1ΔTM2 Synechocystis (C561A/SynEtr1ΔTM2). This amino acid residue is conserved in phytochromes and cyanochromes and has been shown previously to be involved in binding the bilin chromophore required for light sensing in this protein (Song et al., 2011). C561A/SynEtr1ΔTM2 Synechocystis displayed reduced motility in air compared with SynEtr1ΔTM2 (Fig. 2, A and B; Supplemental Fig. S3A). Even though ethylene did not cause a statistically significant increase in the maximum movement of C561A/SynEtr1ΔTM2 cells, as determined by measuring the overall distance moved, it is qualitatively apparent that more cells responded to light in ethylene-treated samples. Thus, ethylene can signal via SynEtr1 independently of light signaling by SynEtr1.

slr1214 Is Needed for Ethylene to Affect Phototaxis

Although SynEtr1 has not yet been shown to be a functional His kinase, mutational studies of slr1213 and slr1214 led to a model that proposes that the output of SynEtr1 involves phosphorelay from SynEtr1 to slr1213 (Narikawa et al., 2011; Song et al., 2011). Phosphorelay from slr1213, in turn, has been suggested to act as a transcriptional activator for another transcription factor, slr1214, that alters phototaxis. To directly test whether slr1214 is required for responses to ethylene, we generated a gene deletion of *slr1214* (Δ1214) as described by Song et al. (2011). The Δ1214 mutant cells had faster phototaxis in response to directional white light, similar to the ΔSynEtr1 cells (Fig. 2E). The mutant failed to respond to ethylene, indicating that the ethylene signaling via SynEtr1 requires slr1214.

Application of Ethylene Affects Pilus Formation

Our results suggest that an underlying aspect of motility may be affected by SynEtr1. Because Synechocystis uses type IV pili to move (Bhaya et al., 1999), we examined the effect of ethylene and SynEtr1 on the transcript abundance of several genes that encode proteins important for pilin formation and function. To do this, we exposed cells to directional white light and used quantitative real-time reverse transcriptase (qRT)-PCR to analyze the transcript levels of *Pilin Polypeptide A1* (*PilA1*), which encodes the major structural protein of type IV pili, and *PilB1* and *PilC*, which encode proteins involved in pilus formation, function, and overall cellular motility (Bhaya et al., 1999, 2000; Yoshihara et al., 2001; Schuergers et al., 2015). Application of 1 μL L^{-1} ethylene had no effect on *PilA1* levels but increased the transcript levels of *PilB1* and *PilC*, suggesting that pili dynamics might be altered (Fig. 3A). *SynEtr1*ΔTM2 Synechocystis had higher transcript levels of *PilA1* and *PilC*. Ethylene had no detectable effect on the transcript levels of these genes in *SynEtr1*ΔTM2 Synechocystis. Thus, ethylene and SynEtr1 may affect phototaxis by altering pilin biogenesis that could lead to alterations in adhesion to surfaces or aggregation, which are factors that affect rates of motility (Burriesci and Bhaya, 2008).

We also used immunoblots to examine the levels of PilA1 protein in both whole cells and isolated pili (Fig. 3B). Equal protein loading for whole cell samples was confirmed with Coomassie Blue-stained samples (Supplemental Fig. S5). In both cases, ethylene did not cause a measurable increase in PilA1 protein. However, SynEtr1ΔTM2 cells showed a large increase in PilA1 protein, suggesting an increase in the number of pili. Unexpectedly, the apparent M_r of PilA1 in SynEtr1ΔTM2 was reduced and corresponds to the M_r of nonglycosylated PilA1 characterized previously (Kim et al., 2009). To more directly test whether ethylene and SynEtr1 affect the number of pili, we negative stained Synechocystis and examined the cells with transmission electron microscopy. We observed that application of ethylene led to a small, but consistent, increase in the number of type IV pili per cell (Supplemental Table S2). Removal of SynEtr1 led to a larger increase in the number of type IV pili that was difficult to quantify (Fig. 3C).

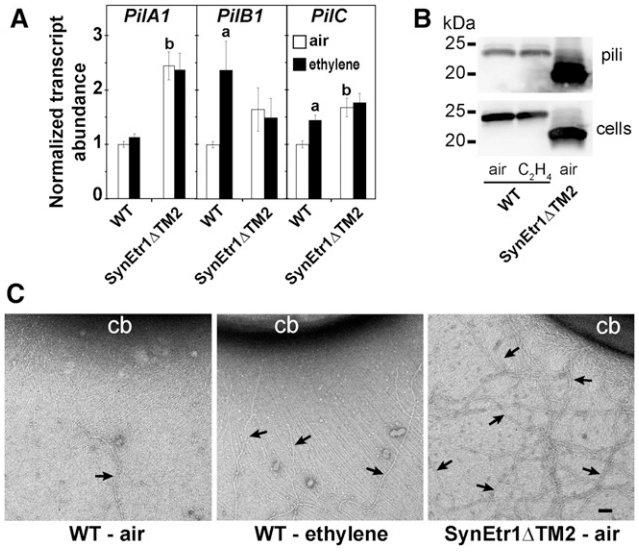

Figure 3. Ethylene and SynEtr1 affect pili formation. A, Synechocystis strains were plated on soft agar plates and exposed to directional white light (30 μmol m^{-2} s^{-1}) for 3 d in ethylene-free air or 1 μL L^{-1} ethylene. Cells were harvested, RNA was extracted, and qRT-PCR was used to analyze the transcript levels of the *PilA1*, *PilB1*, and *PilC* genes. Transcript levels were normalized as described in "Materials and Methods." Data are averages ± SE. [a]Statistical difference caused by the application of ethylene ($P < 0.05$); [b]statistical difference caused by the disruption of SynEtr1 (SynEtr1ΔTM2; $P < 0.05$). B, Equal amounts of Synechocystis cells exposed to directional light were isolated, pili were extracted, and pili proteins were precipitated, solubilized, and electrophoresed with SDS-PAGE. In parallel, total protein was extracted from the cells. Western blotting was conducted and probed with anti-PilA1 antibodies. Equal protein loading for whole cells was confirmed with Coomassie Blue-stained samples (Supplemental Fig. S5). C, Electron micrographs of Synechocystis cells taken from the leading edge of phototaxing colonies and negative stained with uranyl acetate. Arrows mark selected thick type IV pili. Cell bodies (cb) are marked. WT, Wild type. Bar = 100 nm.

SynEtr1 Affects Other Physiological Processes

Type IV pili affect other aspects of Synechocystis biology. In *Pseudomonas aeruginosa*, biofilm formation is linked to type IV pili (O'Toole and Kolter, 1998), suggesting that a similar link might exist in Synechocystis. Because ethylene affects pili, we examined whether ethylene or SynEtr1 affects biofilm formation in Synechocystis. Using Crystal Violet staining of adhered cells, we found that application of 0.3 μL L^{-1} ethylene to wild-type cells led to increased biofilm production (Fig. 4A). By contrast, quantification of Crystal Violet staining of SynEtr1ΔTM2 cells indicated biofilm formation similar to that in wild-type cells, with ethylene having no measurable effect on mutant cells. However, we noted that the mutant cells adhered to glass less than wild-type cells and were more easily rinsed off the glass surface. Visual observation of the cell layer prior to rinsing for Crystal Violet staining suggests that the SynEtr1ΔTM2 cells also may have enhanced biofilm production but that it is more easily disrupted (Fig. 4B) during the Crystal Violet staining process.

Both motility and biofilm formation are linked with extracellular polysaccharides (EPSs) secreted by Synechocystis. Spontaneous cell sedimentation has been employed as a way to screen for EPS mutants (Fisher et al., 2013; Ursell et al., 2013). Therefore, we examined spontaneous cell sedimentation in wild-type and SynEtr1ΔTM2 cells. We were unable to study sedimentation with the application of ethylene because the assay requires samples to be maintained without agitation and the application of ethylene in liquid culture requires bubbling the gas through the liquid. However, in air, SynEtr1ΔTM2 cells sediment slower than wild-type cells (Fig. 4C), indicating that EPSs might be altered by these mutants. To further study this, we used qRT-PCR to analyze the effect of disrupting SynEtr1 on the transcript abundance of *slr1875*, *sll1581*, *sll5052*, and *sll0923* (Fig. 4D), which encode proteins thought to be involved in EPS production (Jittawuttipoka et al., 2013). Disruption of SynEtr1 led to an increase in *slr1875* and a small decrease in *sll5052* ($P < 0.05$). The transcript abundance of *sll1581* also was reduced slightly, but this proved to be not statistically significant ($P = 0.07$). Together, these results suggest that EPS production might be altered by SynEtr1 signaling.

The above results support the idea that ethylene and SynEtr1 signaling affect processes on the surface of Synechocystis cells. We also considered the idea that enhanced movement toward light with the application of ethylene or in SynEtr1ΔTM2 Synechocystis was due to altered photosynthesis. To test this, we used low-temperature fluorescence measurements to examine the PSI-PSII ratio in wild-type and SynEtr1ΔTM2 colonies exposed to directional light (Fig. 4E; Supplemental Fig. S6). Treating wild-type cells with ethylene caused a 27% increase in PSII levels, but this was not statistically significant ($P = 0.1$). In air, PSII levels increased by 44% in SynEtr1ΔTM2 cells ($P = 0.03$). In a single experiment where we treated SynEtr1ΔTM2 cells with ethylene, no increase in PSII levels was observed (data not shown). Thus, ethylene may affect PSII levels via SynEtr1.

Wild-Type and SynEtr1ΔTM2 Cells Affect the Motility of the Cell Population

These results indicate that the accelerated motility in ethylene-treated cells and cells missing SynEtr1 may be largely mediated by an extracellular component such as EPSs or pili or both. If true, we predicted that the phototaxis of a population of cells would be affected by simply mixing wild-type with SynEtr1ΔTM2 cells. By contrast, if phototaxis is affected by an intracellular factor, then the two cell types would separate during phototaxis to form two distinct populations. To distinguish between these possibilities, we mixed these cells in ratios of 1:10, 1:1, and 10:1 (wild type:SynEtr1ΔTM2) and observed phototaxis in response to directional white light (Fig. 5). In support of an extracellular component being altered by ethylene and SynEtr1 to affect phototaxis, wild-type and SynEtr1ΔTM2 cells did not

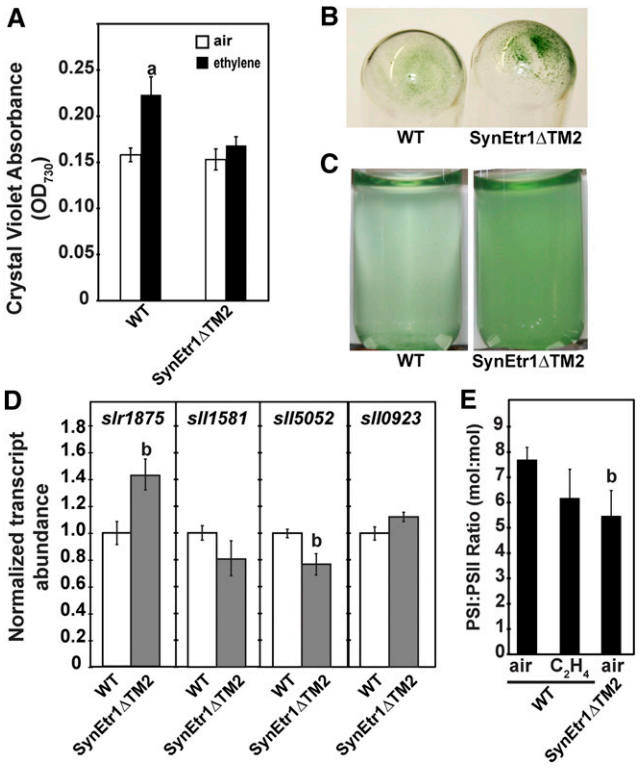

Figure 4. Ethylene and SynEtr1 affect other responses. A, Biofilm formation was assayed in Synechocystis cells exposed to 0.3 μL L^{-1} ethylene or ethylene-free air for 4 d. Quantification of biofilm formation was carried out by measuring the staining of attached cells with Crystal Violet. Data are means ± SD. OD_{730}, Optical density at 730 nm. [a]Statistical difference caused by the application of ethylene ($P < 0.05$). B, Photographs of wild-type (WT) and SynEtr1ΔTM2 Synechocystis cells adhering to glass tubes prior to rinsing for the Crystal Violet assay. C, Photographs of cultures of wild-type and SynEtr1ΔTM2 Synechocystis cells kept static for 4 d prior to imaging. D, Wild-type (white bars) and SynEtr1ΔTM2 (gray bars) Synechocystis cells were plated on soft agar plates and exposed to directional white light (30 μmol m^{-2} s^{-1}) for 3 d in ethylene-free air. Cells were harvested, RNA was extracted, and qRT-PCR was used to analyze the transcript levels of the *slr1875*, *sll1581*, *sll5052*, and *sll0923* genes. Transcript levels were normalized as described in "Materials and Methods." Data are averages ± SE. E, The molar ratio of PSI to PSII based on low-temperature fluorescence measurements was determined (Supplemental Fig. S6). Measurements were conducted on cells from phototaxing colonies in response to directional white light (30 μmol m^{-2} s^{-1}) for 3 d in the presence or absence of 1 μL L^{-1} ethylene. [b]Statistical difference caused by the disruption of SynEtr1 (SynEtr1ΔTM2; $P < 0.05$).

form two distinct populations during phototaxis. Rather, the population of cells showed phototaxis patterns intermediate between wild-type and SynEtr1ΔTM2 cells that correlated with the ratios used. In other words, excess wild-type cells led to less motility of the colony. By contrast, the motility of the population of cells increased as the relative levels of SynEtr1ΔTM2 cells in the colony increased. However, even in the colonies with a 10:1 ratio of wild-type to SynEtr1ΔTM2 cells, the motility of the mixture increased over that of wild-type cells alone.

DISCUSSION

Ethylene receptors are well studied in plants, leading to models for ethylene binding and signal transduction (Shakeel et al., 2013; Lacey and Binder, 2014). The current models for ethylene binding to plant receptors posit that ethylene receptors are homodimers that coordinate a Cu(I) that is required for binding. The copper cofactor is predicted to be coordinated in the binding pocket by several highly conserved amino acids. In this study, we show that SynEtr1 directly binds ethylene, and our results mutating SynEtr1 suggest that the binding pocket in this receptor is similar to the binding pocket in plant receptors. Additionally, the application of ethylene modulates important aspects of Synechocystis biology, including motility, pili biosynthesis, photosynthesis, and biofilm formation via SynEtr1. Disruption of SynEtr1 causes both an increase in the transcript abundance of *PilA1* and an apparent deglycosylation of PilA1. Thus, PiLA1 protein levels may be regulated by changes in both synthesis and degradation. SynEtr1 also affects spontaneous cell sedimentation. The facts that SynEtr1 saturably binds ethylene with high affinity and SynEtr1 is required for ethylene responses support the conclusion that SynEtr1 is a functional ethylene receptor. Therefore, ethylene receptors are not confined to plants. Prior research has shown that SynEtr1 is a photoreceptor involved in phototaxis (Ulijasz et al., 2009; Narikawa et al., 2011; Song et al., 2011). Thus, SynEtr1 is likely to be a receptor for both light and ethylene, making it a bifunctional receptor for two very different signals.

It is likely that many of the responses altered by ethylene and SynEtr1 are linked. For instance, motility, biofilm formation, and cell sedimentation of Synechocystis are dependent on both type IV pili and EPSs (Bhaya et al., 1999, 2000; Burriesci and Bhaya, 2008; Jittawuttipoka et al., 2013). Thus, ethylene may alter

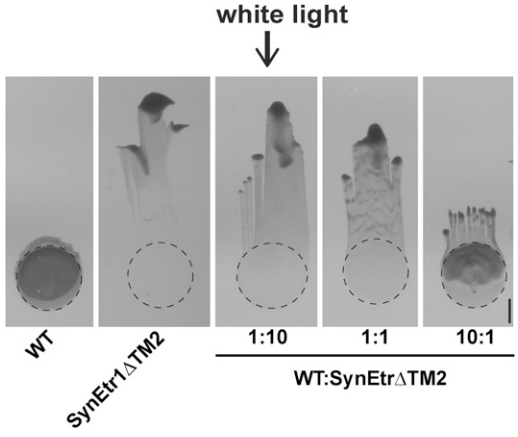

Figure 5. Mixing wild-type (WT) and SynEtr1ΔTM2 cells affects phototaxis of the cell population. Colonies of Synechocystis cells were spotted on soft agar plates and exposed to directional white light (30 μmol m^{-2} s^{-1}) in ethylene-free air. At the end of 4 d, images were acquired of wild-type and SynEtr1ΔTM2 cells and mixtures at the indicated wild type:SynEtr1ΔTM2 ratios. Dotted circles mark the initial positions of spots. Bar = 2 mm.

various physiological responses through alterations in extracellular characteristics via changes in EPSs and type IV pili. However, we cannot rule out that intracellular changes, such as alterations in PSII levels, also affect physiological responses. Our results mixing wild-type and SynEtr1ΔTM2 cells favor the idea that extracellular changes mediate the major effects of ethylene that we observed on phototaxis. It is unclear whether the changes in PSII levels are a primary or secondary effect of SynEtr1 signaling, because it has been observed previously that the accumulation of prepilin proteins can affect PSII levels by altering the translocation of proteins necessary for PSII formation (Linhartová et al., 2014). Thus, the alterations we see in PSII levels may be an indirect effect of this signaling system that is related to changes in pilin protein levels.

We studied the function of SynEtr1 by both disrupting the coding sequence of the second transmembrane helix of *SynEtr1* (SynEtr1ΔTM2) and deleting the *SynEtr1* gene (ΔSynEtr1) using constructs described previously (Rodríguez et al., 1999; Song et al., 2011). The SynEtr1ΔTM2 cells consistently had faster motility toward white light compared with ΔSynEtr1 cells. Interestingly, Narikawa et al. (2011) demonstrated that another disruption of *SynEtr1* in the coding sequence for the first transmembrane helix also resulted in enhanced motility. However, those mutants had enhanced negative phototaxis. It is likely that a contributing factor in the observed variations in response was the use of different *Synechocystis* strains (*Synechocystis* sp. PCC-N and *Synechocystis* sp. PCC-P) by Narikawa et al. (2011) that contain known genetic differences from Synechocystis (Trautmann et al., 2012). Additionally, the variations in response could be caused by differences in the location of the disruption. The mechanism for the differences between SynEtr1ΔTM2 and ΔSynEtr1 has yet to be determined. One possibility is that the SynEtr1ΔTM2 disruption results in a truncated SynEtr1 missing the first two transmembrane domains that constitutively signal to affect motility. It is also possible that the downstream signaling components, slr1213 and slr1214, are differentially affected by each construct because of differences in transcription downstream of the mutations. Despite these discrepancies between SynEtr1ΔTM2 and ΔSynEtr1, ethylene failed to alter motility in either mutant, supporting the idea that SynEtr1 is an ethylene receptor that is required for responses to ethylene.

It is unknown whether light or ethylene or both act by regulating the His kinase activity of SynEtr1. A bioinformatics analysis of the SynEtr1 His kinase domain showed that the His residue predicted to be autophosphorylated is located in a noncanonical position, raising the possibility that this protein has a noncanonical output (Narikawa et al., 2011). However, mutations in the conserved Asp residues that are predicted to be phosphorelay sites in slr1213 and slr1214 (the predicted downstream targets of SynEtr1) led to alterations in phototaxis, suggesting that His kinase plays a role in SynEtr1 function (Song et al., 2011). Thus, it is an open question how SynEtr1 signals. Despite this uncertainty about SynEtr1 output, we found that removing SynEtr1 and applying ethylene to wild-type cells have similar effects. This supports a model where SynEtr1 inhibits downstream events and ethylene inhibits the output of the receptor. This inhibition of the receptor by ethylene is similar to the inverse-agonist model for ethylene receptor signaling proposed for plants (Hall et al., 1999), suggesting a conservation of function within the ethylene-binding domain. Our results also support a model where signaling from SynEtr1 requires slr1214, which is in agreement with models developed for this pathway from other studies (Narikawa et al., 2011; Song et al., 2011).

The ecophysiological role of ethylene for Synechocystis remains an open question. Unlike plants and some bacteria, Synechocystis is unable to biosynthesize ethylene (Guerrero et al., 2012; Ungerer et al., 2012). Thus, it must encounter ethylene in the environment from nearby organisms that biosynthesize ethylene or from abiotic sources. Ethylene levels found in nature can exceed the levels used in this study, supporting a role for ethylene in Synechocystis physiology (Abeles et al., 1992). Ethylene is photochemically produced when organics dissolved in aqueous environments are exposed to sunlight (Swinnerton and Linnenbom, 1967; Wilson et al., 1970; Swinnerton and Lamontagne, 1974; Ratte et al., 1993, 1998). Additionally, these prior studies show that ethylene can diffuse and that ethylene levels vary depending upon various environmental conditions, such as light levels and depth in the water column. Interestingly, abiotically produced ethylene can affect the growth of plants (Buer et al., 2000, 2003). These observations lead to the hypothesis that Synechocystis may use both light and ethylene as cues to move to regions where light levels are optimal for photosynthesis and survival. In this model, ethylene enhances motility so that the cells can move more quickly to optimal lighting. Additionally, since the application of ethylene causes Synechocystis to respond to blue light, it is possible that ethylene broadens the wavelength response range to aid in this process. Synechocystis is known to establish symbiotic relationships (Rai, 1990), raising the alternative possibility that ethylene produced by a host is an interspecies signaling molecule during the process of forming or maintaining this relationship.

In summary, we have characterized an ethylene receptor from a cyanobacterium that affects the physiology of Synechocystis. This study shows that ethylene perception evolved prior to green plants. However, even though functional ethylene receptors originated prior to the evolution of green plants, the remainder of the ethylene signaling pathway in green plants is from another source (Ju et al., 2015). The fact that potential ethylene receptors exist in other bacteria opens up novel avenues of research to determine the signaling pathways and roles for ethylene in a variety of bacteria. The observation that many soil proteobacteria that form commensal and mutualist associations with plants also contain putative ethylene receptors suggests the interesting possibility that ethylene acts as a gaseous signal from the plants to affect colonization by bacteria.

MATERIALS AND METHODS

Cloning and Site-Directed Mutagenesis of Codon-Optimized *SynEtr1* for Expression in *Pichia pastoris*

GS115 *P. pastoris* was used for all experiments. *P. pastoris* was grown in yeast peptone dextrose medium at 30°C. Codon-optimized Synechocystis (*Synechocystis* sp. PCC 6803) *SynEtr1* for expression in *P. pastoris* was obtained from GenScript. From the full-length codon-optimized *SynEtr1*, a truncated version (the first 390 bp) of the gene, corresponding to the coding region for the putative ethylene-binding domain of SynEtr1, was amplified by PCR with a 5′ *Eco*RI restriction site and a 3′ *Xho*I restriction site using 5′-GAATTCATGGCTATTACAGCTTTTAC-3′ and 5′-CTCGAGCTCGGTAGGGGACTTCAA-3′ as primers. This PCR fragment was then subcloned into a pGEM-T vector and transformed into DH5α *Escherichia coli*. Plasmids were then isolated using the Promega PureYield Plasmid Mini Prep system. The truncated, codon-optimized *SynEtr1* was excised at the *Eco*RI and *Xho*I sites and ligated into the *P. pastoris* expression vector, pPICZA, containing a GST tag at the 3′ end of the gene, with subsequent transformation into DH5α *E. coli*. Plasmids were isolated, and successful ligation was confirmed by PCR and sequencing. Plasmids were then transformed into *P. pastoris* as described previously (McDaniel and Binder, 2012).

Site-directed mutagenesis was performed on pPICZA plasmids containing codon-optimized truncated *SynEtr1*. Primers were designed using DNASTAR Lasergene. The primers used for this were as follows (lowercase letters denote the positions of point mutations): *C77A*, 5′-CTTTTCTCTACTTTTATTTTGgctTGCG-GAACCTCCCACTTTTTCG-3′ (forward) and 5′-CGAAAAAGTGGGAGGTTCCG-CAagcCAAAATAAAAGTAGAGAAAAG-3′ (reverse); *C78A*, 5′-CTTTTCTC-TACTTTTATTTTGTGTgctGGAACCTCCCACTTTTTCG-3′ (forward) and 5′-CGAAAAAGTGGGAGGTTCCagcACACAAAATAAAAGTAGAGAAAAG-3′ (reverse); *C78S*, 5′-CTTTTCTCTACTTTTATTTTGTGTtccGGAACCTCCCACT-TTTCG-3′ (forward) and 5′-CGAAAAGTGGGAGGTTCCggaACACAAAA-TAAAAGTAGAGAAAAG-3′ (reverse); and *H82A*, 5′-TGTGTTGCG-GAACCTCCgcgTTTTTCGATATTATC-3′ (forward) and 5′-GATAATATCG-AAAAAcgcGGAGGTTCCGCAACACA-3′ (reverse). The entire plasmid was amplified by ExTaq using primers with specific point mutant modifications. Following PCR, the plasmids were transformed into DH5α *E. coli* and isolated, and point mutations were confirmed by sequencing. Confirmed plasmids were then transformed into *P. pastoris*.

Expression of SynEtr1 in *P. pastoris* and Whole-Cell Ethylene-Binding Assays

SynEtr1 was expressed in *P. pastoris* based on the protocol described in the Invitrogen *P. pastoris* manual. Copper sulfate (5 μM) also was added to the expression medium. Following a 48-h induction with methanol, cells were harvested and washed once with water. Whole-cell ethylene-binding assays were then performed on 3 g (wet weight) of *P. pastoris* as described previously (Schaller and Bleecker, 1995).

Synechocystis Strains and Growth Conditions

Motile Synechocystis was obtained from the Pasteur Institute and grown on 1% modified BG-11 (Rippka et al., 1979) plates containing 100 nM $CuSO_4$ in continuous light (30 μmol m^{-2} s^{-1}) at 24°C. Kanamycin, spectinomycin, and streptomycin were added to growth plates as needed for selection of *SynEtr1* knockout and rescue lines. Nonmotile *Synechocystis* sp. ATCC 27184 was obtained from the laboratory of Anthony Bleecker and was originally from the American Type Culture Collection.

Generation and Confirmation of *SynEtr1* Disruption, Deletion, and Rescue Lines

SynEtr1ΔTM2 mutants disrupting the *SynEtr1* gene in the gene encoding the second transmembrane helix were generated using the construct described previously (Rodríguez et al., 1999). Colony PCR was performed using a *SynEtr1* 5′-CTACTTTTTGCGGAAGCGCCA-3′ (forward) and 5′-CGGTCTTCCACTC-TTTTTTCAAG-3′ (reverse) primer pair bracketing the gene insert. An increase in size of the PCR product indicated the presence of the kanamycin resistance gene insert. ΔSynEtr1 and Δslr1214 deletion mutants were generated as described previously (Song et al., 2011). Colony PCR was used to confirm these mutants (Supplemental Fig. S4C) with primers used to generate the deletions.

The pUR self-replicating vector was used to express *SynEtr1* in the SynEtr1ΔTM2 background (Wiegard et al., 2013). The pUR vector confers both kanamycin and spectinomycin resistance. Because SynEtr1ΔTM2 Synechocystis also lacks slr1213, a PCR fragment containing both *SynEtr1* and *slr1213* (*SynEtr1-1213*) was cloned into pUR. *SynEtr1-1213* was first subcloned into a pGEM-T vector with a 5′ *Nde*I restriction site and a 3′ *Bam*HI restriction site using *SynEtr1*_sub 5′-CGCATATGATGGCAATCACCGCATTTACC-3′ (forward) and 5′-GAGGATCCCTAGCGACTACGATATTCTTTC-3′ (reverse) primers. Following cloning and transformation into DH5α *E. coli*, the pGEM-T vectors containing *SynEtr1-1213* were isolated.

Site-directed mutagenesis was performed as described above with primers designed in DNASTAR and ExTaq used in PCR. To generate the C78S mutation, 5′-CCTAATATTATTTTCCTGTTTAGTACTTTTATTTTATGCtctGGC-3′ (forward) and 5′-GCCagaGCATAAAATAAAAGTACTAAACAGGAAAATAA-TATTAGG-3′ (reverse) primers were used. To generate the C561A mutation, 5′-GTCCCATCTTAGGCAAgcaCACATTGACTTTCTTGC-3′ (forward) and 5′-GCAAGAAAGTCAATGTGtgcTTGCCTAAGATGGGAC-3′ (reverse) primers were used. Following successful ligation of wild-type *SynEtr1-1213* and site-directed mutants into pUR, the plasmid was transformed into S-17 *E. coli* for biparental conjugation.

For biparental mating, 3 mL of S-17 *E. coli* containing *SynEtr1-1213* was grown overnight in Luria Broth (LB) medium at 37°C. The cells were then harvested, washed twice with 2 mL of fresh LB medium, and resuspended in 200 μL of BG-11. SynEtr1ΔTM2 Synechocystis (0.5 mL) also was harvested and resuspended in 1 mL of BG-11. The cyanobacterial cells were then combined with the S-17 *E. coli* cells. This mixture was harvested and resuspended in 200 μL of BG-11. This mixture was then plated on sterile nitrocellulose filter paper placed on BG-11 + 5% LB agar plates. These plates were incubated in low light at 30°C for 24 h. The filter paper was transferred to 1% BG-11 plates containing low levels of both kanamycin and spectinomycin. The filter paper was then transferred every few days to BG-11 plates containing higher antibiotic concentrations, until colonies were visible.

Colonies were selected and streaked on fresh BG-11 plates, and transformants were validated by colony PCR. One validation was to use the *SynEtr1* forward and reverse primer pair bracketing the gene insert, which resulted in two products. The smaller product corresponded to the transformed gene and the larger product corresponded to the knockout gene. We also confirmed successful transformation using a *PpetJ* primer (5′-CGATCGCCATCGGCAC-CATGAAAC-3′; forward) to the *PpetJ* promoter of the pUR vector paired with the *SynEtr1*_sub reverse primer detailed above.

Phototaxis Assays

Phototaxis assays were performed on 0.4% agar-modified BG-11 plates with 100 nM $CuSO_4$. All assays were performed at 28°C at a fluence rate of 30 μmol m^{-2} s^{-1} unless specified otherwise. In some cases, monochromatic light arrays were used. These assays also were performed at 28°C at the indicated fluence rates using light-emitting diode arrays from Quantum Devices for red (λ_{max} = 672 nm), green (λ_{max} = 525 nm), and blue (λ_{max} = 462 nm) light and a UV-A F20T8/BLB fluorescent light (λ_{max} = 365 nm) from CH Lighting. Synechocystis cells were taken from plates and resuspended to a dense concentration of fresh BG-11. Ten microliters of cells was spotted onto plates and placed under diffuse white light (30 μmol m^{-2} s^{-1}) for 24 h. The plates were then wrapped, allowing for directional light exposure at the indicated fluence rate, and placed in flow-through chambers with either constant hydrocarbon-free air or 1 μL L^{-1} ethylene circulation. Phototaxis was allowed to progress for up to 2 weeks as indicated in each figure. Images of each plate were acquired using a flatbed scanner. Colony movement was analyzed and measured using ImageJ. Movement was quantified as the maximum distance moved from the leading edge of the original position of the colony to the position of maximum movement after exposure to directional light. In all conditions, phototaxis assays were repeated at least three times with at least three colonies in each experiment. We noted that the amount of time that cells were maintained in culture affected motility. Therefore, when conducting these assays, we age matched our control cells with mutants and transformants. In all cases, ethylene stimulated motility in wild-type cells. Initial observations of the effects of ethylene and disrupting SynEtr1 were made on Synechocystis obtained from Iwani Suzuki and yielded similar results to the strain obtained from the Pasteur Institute.

Biofilm Formation and Spontaneous Cell Sedimentation Assays

For both biofilm and sedimentation assays, Synechocystis cells were grown in liquid culture to late log phase. The cells were then harvested and resuspended in fresh BG-11 to a final optical density at 730 nm = 0.1 for biofilm analysis and an

optical density at 730 nm = 1 for cell sedimentation assays. Biofilm formation by Synechocystis cells was then assessed using the methods of Fisher et al. (2013). Briefly, 2 mL of cells was divided into aliquots in glass test tubes and maintained for 4 d in the absence or presence of 0.3 μL L^{-1} ethylene. At this time, we first visually inspected the layer of cells at the bottom of the tube and then assayed biofilm formation using Crystal Violet. Cell sedimentation assays were performed as described by Jittawuttipoka et al. (2013). For this, 2 mL of cells was divided into aliquots in test tubes and maintained with no agitation for 4 d. The extent of sedimentation was then evaluated visually. All samples were replicated in triplicate.

RNA Isolation, Complementary DNA Synthesis, and qRT-PCR

For gene expression analysis, RNA was initially extracted from Synechocystis cells exposed to directional light for 3 d on 1% modified BG-11 plates in the presence of air or 1 μL L^{-1} ethylene. Following a 3-d directional light treatment, cells were removed from the plate and added to 1 mL of fresh BG-11. The cells were then harvested and resuspended in 1 mL of Trizol. The cells were next incubated at 95°C for 5 min followed by 5 min on ice. Phase separation was performed by the addition of 200 μL of chloroform. The cells were vortexed for 30 s. After 5 min, the cells were centrifuged for 15 min at 12,000*g* at 8°C. The aqueous phase was then transferred to a new tube, and RNA was precipitated by the addition of an equal volume of isopropanol. Following a 5-min rest at room temperature, the precipitated RNA was pelleted by centrifugation (12,000*g*) for 10 min at 8°C. The RNA pellet was then washed twice by resuspension in 75% ethanol with subsequent centrifugation (8,000*g*) for 5 min at 8°C. The washed RNA was then resuspended in 100 μL of diethyl pyrocarbonate-treated water. Eight micrograms of RNA was then treated with DNase for 30 min at 37°C using the TURBO DNA-free kit from Invitrogen. Following inactivation of the DNase by use of the inactivation reagent supplied with the kit, the RNA was further washed using the Spectrum Plant Total RNA Kit from Sigma and eluted in 50 μL of diethyl pyrocarbonate-treated water.

For complementary DNA (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 30 μL with reagents at concentrations suggested by the manufacturer. Random hexamers were used for priming. For negative control samples lacking reverse transcriptase, water was used in place of the reverse transcriptase. Following synthesis, the cDNA was diluted at a ratio of 1:4.

Primers for quantitative PCR (qPCR) were designed using the qPCR primer design software on the GenScript Web site. These were 5′-CCAACTTGACCCAAGAGGTT-3′ (forward) and 5′-AATCGAACTGCTGGTTGTTG-3′ (reverse) to quantify *PilA1*; 5′-CTCCATCGACATGAATCTGG-3′ (forward) and 5′-TTCCTTCCTGGAGAGCCTTA-3′ (reverse) to quantify *PilB1*; 5′-GAAGCCATGAGTCCAGAACA-3′ (forward) and 5′-TTTCCAGGAATTCGAGGTTT-3′ (reverse) to quantify *PilC*; 5′-CATTTCCATGATGATTCCCA-3′ (forward) and 5′-TCCCTGCTCCGGTAATTAAG-3′ (reverse) to quantify *slr1875*; 5′-GCGAATATCTGCTTGGGATT-3′ (forward) and 5′-ATCACCGAAGTGAGTGGTGA-3′ (reverse) to quantify *sll1581*; 5′-TTATCATGGGCAATTCCTGA-3′ (forward) and 5′-ACAGCGACCTTAAATTTGGC-3′ (reverse) to quantify *sll5052*; 5′-TAATTTCGTTGAGGCGTGAG-3′ (forward) and 5′-TTAATGACCAGTTGCCCAAA-3′ (reverse) to quantify *sll0923*; and 5′-GCGGATTTAATTGAGTTGGG-3′ (forward) and 5′-GCACATCATCCAACTGACC-3′ (reverse) to quantify transcript levels of the Trp synthase gene (*TrpA*). *TrypA* was used as an internal control and has been used previously as a housekeeping gene (Zhang et al., 2008).

qPCR was performed using the Bio-Rad iQ5 Real-Time PCR Detection System. Each reaction was 10 μL total with 5 μL of Ssofast EvaGreen Supamix from Bio-Rad, 4 μL of 1:4 diluted cDNA (1:10 final dilution), and 1 μL of a 10 μM forward and reverse primer mix (0.5 μM each final primer concentration). Cycle times were as follows: denaturation at 95°C for 2 min, followed by 40 cycles of 95°C for 10 s and 58°C for 20 s. Transcript amounts were normalized to levels of *TrpA* as the internal standard using the method of Livak and Schmittgen (2001) for each cell line under each condition. Data were then normalized to gene transcript levels in wild-type cells maintained in air. Each gene was analyzed with two biological replicates, with each replicate done with three technical replicates. Average values ± SE are shown for all experiments.

Pili Protein Analyses

Pili were isolated from an equal amount of cells from colonies of phototaxing Synechocystis according to methods described previously (Nakasugi et al., 2006). Total protein also was extracted from these phototaxing Synechocystis colonies. In both cases, proteins were separated by SDS-PAGE. PilA1 protein levels were analyzed with western blots using a 1:10,000 dilution of anti-PilA1 antibodies obtained from Dr. Roman Sobotka (Linhartová et al., 2014). A horseradish peroxidase-conjugated secondary antibody was used, and detection with luminol (Bio-Rad) was performed. Samples run in parallel were stained with Coomassie Blue to visualize proteins.

Electron Microscopy

Samples of cells from the leading edge of phototaxing colonies were stained with 1% (w/v) uranyl acetate for 1 min, and images were obtained using a Zeiss Libra 200 MC electron microscope. We examined at least 10 cells from three separate colonies for each condition.

Low-Temperature Fluorescence Measurements

To determine PSI-PSII ratios, Synechocystis cells from phototaxing colonies were harvested and analyzed with chlorophyll low-temperature (77 K) fluorescence at an excitation wavelength of 440 nm according to methods described previously (Murakami, 1997; Kiley et al., 2005; Iwuchukwu et al., 2010).

Statistics

Data were analyzed with Student's *t* test and considered statistically different at $P < 0.05$.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Phylogenetic tree of predicted ethylene-binding domains from nonplant genomes.

Supplemental Figure S2. Additional examples of ethylene-stimulated movement.

Supplemental Figure S3. Closeup images of phototaxing colonies of Synechocystis.

Supplemental Figure S4. Colony PCR confirmation of disruption and deletion mutants.

Supplemental Figure S5. Coomassie Blue-stained SDS-PAGE results of proteins from whole cells.

Supplemental Figure S6. Low-temperature fluorescence measurements.

Supplemental Table S1. Predicted ethylene-binding proteins and domain structures in nonplant genomes.

Supplemental Table S2. Effect of ethylene on the number of type IV pili per cell.

ACKNOWLEDGMENTS

We thank Annegret Wilde for sharing the pUR vector, Liz Fozo for sharing the pCR2.1 cloning vector, and Roman Sobotka for anti-PilA1 antibodies; Cidney Allen, Khoa Nguyen, M. Blaine Stalans, Christopher Gilmartin, Jalissa Wynder, Zhou Li, Sara Lemmonds, and Daniel Degennaro for technical assistance; Gladys Alexandre, Devaki Bhaya, Barry Bruce, Tessa Burch-Smith, Liz Fozo, Andreas Nebenführ, Iwani Suzuki, Annegret Wilde, and Joe Williams for helpful information; the University of Tennessee Advanced Microscopy and Imaging Center for electron microscope use and technical assistance; and Caren Chang, John Clay, and Eric Schaller for feedback on the article.

Received April 26, 2016; accepted May 28, 2016; published May 31, 2016.

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