A novel activation domain is essential for CIA5-mediated gene regulation in response to CO_2 changes in *Chlamydomonas reinhardtii*

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ABSTRACT

The inducible CO_2 concentrating mechanism (CCM) of microalgae is essential for their acclimation to highly variable aquatic inorganic carbon levels. The key transcriptional regulator, CIA5, affects expression of thousands of genes in *Chlamydomonas reinhardtii*, but the molecular characteristics of this important protein are poorly understood. This study identifies a functional activation domain in CIA5 and demonstrates the functionality of a mini-CIA5 including only the Zinc-binding domain and the identified activation domain. A highly conserved 130aa region from CIA5 exhibits auto-activation in yeast and appears responsible for the markedly slow migration of CIA5 when analyzed by SDS-PAGE. This 130aa region or either half of this region also effectively replaced the activation domain of a modified designer Transcription Activator-like Element (dTALE) in targeted activation of an endogenous Chlamydomonas gene. Additionally, a mini-CIA5 combining the conserved zinc-binding domain with the 130aa putative activation domain complemented the growth phenotype of the *cia5* mutant and triggered CO_2-regulated gene expression patterns similar to wild-type cells or *cia5* complemented with the full-length *CIA5*. Although the mini-CIA5 complementation did not fully restore wild-type growth rates or full gene induction/repression amplitudes, especially in very low CO_2, this newly identified activation domain combined with the previously described zinc-binding domain are demonstrated to be the key essential components of CIA5 that permit rapid CIA5-mediated responses to changes in CO_2 concentrations.

Keywords:

CO_2 concentrating mechanism (CCM), CIA5, Activation Domain, recombinant mini-CIA5, Chlamydomonas

1. Introduction

Carbon dioxide (CO_2) serves both as the substrate for photosynthesis and as an important signal to regulate plant growth and development. Variable CO_2 concentrations can have significant effects on photosynthesis, growth and productivity of plants and other photosynthetic organisms. Although atmospheric CO_2 has increased from a preindustrial concentration of about 280 ppm to a globally averaged concentration of approximately 400 ppm at present (http://co2now.org/), this CO_2 concentration is still limiting to photoautotrophic growth of most land plants and photosynthetic microorganisms. This is because of the inefficiency of the enzyme Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), which has both an oxygenase and a carboxylase function, as well as a low affinity for CO_2 [1, 2].

A number of photosynthetic organisms have developed strategies to increase the concentration of CO_2 surrounding Rubisco to improve its function, including the well-known C4 photosynthesis and Crassulacean Acid Metabolism (CAM) pathways in terrestrial vascular plants [3]. In contrast, some aquatic photosynthetic microorganisms accomplish this through induction of a different type of CO_2-concentrating mechanism (CCM) when photosynthesis is limited by inorganic carbon (Ci; i.e. dissolved CO_2, and HCO_3^-). Much knowledge regarding the mechanisms of the microalgal CCM has been gained from the well-studied eukaryotic microalga, *Chlamydomonas reinhardtii* [4-8] (hereafter,

Chlamydomonas). When Ci becomes limiting, the CCM becomes activated and acts via Ci uptake systems located in both the plasma membrane and the chloroplast envelope to increase internal Ci accumulation. Various carbonic anhydrases function to dehydrate accumulated HCO_3^- and provide elevated internal CO_2 concentrations in the near vicinity of Rubisco [4, 5, 7-9].

Even though the Chlamydomonas CCM has been extensively studied for at least three decades, we still know little about the CO_2 sensing process, the induction (and/or de-repression) of the CCM or the final acclimation to limiting (or excess) CO_2. In Chlamydomonas, CCM induction appears to be regulated by the transcription regulator, CIA5, the so-called "master regulator" of the CCM. [10-13]. A UV- induced mutant, *cia5*, was first identified as a slow growing mutant in ambient CO_2 concentration [14]. This mutant can grow under high CO_2 and grows more slowly than the wild type in low CO_2. However, it cannot grow in very low CO_2 (i.e., <100ppm) and appears to completely lack the changes in gene expression associated with acclimation to low CO_2 [15]. Further studies show that almost all identified Low CO_2 Inducible (LCI) genes remain unchanged when *cia5* mutant is exposed to limiting CO_2 [14-18]. The defective gene in *cia5*, *CIA5* (also known as *CCM1*), was identified independently by two research groups as encoding a predicted 698 amino acid hydrophilic protein [12, 13].

CIA5/CCM1 has been proposed to be a putative transcription factor or transcription co-activator; it has two non-typical zinc-binding domains, which may comprise a DNA-binding motif typical of transcription factors. It was confirmed that 2 atoms of zincs can bind to the N-terminal domain of each CIA5 molecule, and that, if mutated to perturb the putative zinc binding sites (H54Y, C77V and C80V), the zinc binding ability is lost [19]. CIA5 also

contains a Gln-rich repeat that may allow interactions with and/or activation of other transcription factors, and a prominent Gly-rich region, but the function of these regions has not been experimentally determined. The 72 kD CIA5 protein that regulates the Chlamydomonas CCM migrates as an approximately 90-100 kD during SDS PAGE [11, 19]. This suggested either aberrant migration of CIA5 or potential posttranslational modification of CIA5.

Aside from being a critical upstream regulator of the CCM and other low CO_2 acclimation responses (and likely requiring posttranslational modifications during high and low CO_2 concentration transitions), the details of CIA5 function remain undiscovered. We know very little about sequences recognized by its putative DNA binding domain or the downstream genes it directly regulates [17]. Two studies exploring the impact of the *cia5* mutation on the transcriptome identified a massive impact of CIA5/CCM1 and CO_2 on the transcriptome and revealed an array of gene clusters with distinctive expression patterns that provide insights into the regulatory interaction between CIA5 and CO_2 [17, 18]. Individual gene clusters responded primarily to CIA5, to CO_2, or to an interaction between the two factors [17]. In this study, we found that CIA5 has transcriptional activation activity both in yeast and in Chlamydomonas. We also discovered that the identified acidic activation domain also is responsible for abnormal SDS-PAGE migration of *CIA5*. A recombinant mini-CIA5 containing the minimal activation domain and the putative zinc-binding domain of CIA5 successfully complemented the *cia5* mutant. The identification of a functional CIA5 activation domain and the characterization of regions responsible for abnormal migration during SDS-PAGE, together with the construction of a functional mini-CIA5, provide a solid foundation for further work to better define the detailed mechanisms of CIA5 function.

2. Materials and methods

2.1. Cell strains and culture conditions

Chlamydomonas strain CW10 (cc849) was obtained from the Chlamydomonas Resource Center (University of Minnesota, St. Paul, MN). Media and growth conditions for Chlamydomonas strains have been previously described [20]. All strains were maintained on plates containing CO_2 minimal medium and kept in high CO_2 (air enriched with 5% vol/vol CO_2) chambers at room temperature, under continuous illumination (50 μmol photons m^{-2} s^{-1}). Liquid cultures for experimental use were grown on a gyratory shaker (180 rpm) under aeration in white light (approximately 100 μmol photons m^{-2} s^{-1}). For experiments in which cells were shifted from high to limiting CO_2 (low CO_2, 350-400 ppm or very low CO_2, 50-100 ppm) conditions, cells were cultured in CO_2 minimal medium aerated with 5% CO_2 to a density of $\approx 2\times10^6$ cells/ml and then shifted to aeration with the appropriate limiting CO_2 for various times. Very low CO_2 was obtained by mixing normal air with compressed, CO_2-free air.

For spot tests of cell growth, actively growing cells were 5-fold serially diluted to similar cell densities in minimal medium, spotted (5 μL/spot) onto minimal agar plates, and grown in various CO_2 concentrations for approximately 9 days.

For liquid-culture growth experiments, cells inoculated from agar plates were cultured in CO_2 minimal medium aerated with 5% CO_2 for about 2 days, then centrifuged at 1300 rpm for 5 min, and an appropriate aliquot used to inoculate experimental cultures at a starting

density of 1×10^5/mL in a fresh 50 ml culture. Liquid cultures were grown in 250 ml flasks on a gyratory shaker (180 rpm) under aeration (with bubbling) in 3 CO_2 conditions: high CO_2, air-level CO_2 and very low CO_2, and were sampled daily for both cell density and chlorophyll concentration. Because Sager and Granick found OD_{750} of cell cultures to be linear over a range of 2×10^5 to 1×10^7 cells/ml [21] and because, at this wavelength, chlorophyll absorbance does not interfere, optical density at 750 nm (OD_{750}) was used to monitor algal cell density. Growth assays were performed in clear, flat-bottom 96-well microtiter plates monitored at 750 nm using a Synergy 2 Multi-Mode Plate Reader (BioTek Instruments, Inc) as described by Bernd and Cook [6].

2.2. Modified Yeast two-hybrid and LacZ assay

The yeast two-hybrid procedure was performed in accordance with the protocol for the HybriZAP-2.1 XR library construction kit (Stratagene). The *Eco*RI - *Sal*I fragments containing the full length coding DNA sequence (CDS) or partial CDS of *CIA5* were subcloned into pGBKT7 (Clontech) to make bait constructs. Yeast (AH109) cells transformed with indicated constructs were grown in SD media lacking Trp or Trp and His. Putative positive clones were used for LacZ assays using the X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; Sigma) colony-lift filter assay performed following the manufacturer's instructions (Clontech).

2.3. Chlamydomonas dTALE construction and colorimetric assay

The dTALE was constructed as previously described using an altered TAL plasmid pSKavrXa7delta and a Chlamydomonas expression vector pDW2177 [22]. Only the pDW2177ARS1 construct was used for this study. This dTALE construct was cut with *Bbv*CI and *Xba*I to remove the activation domain [23], and CIA5 activation domain (AD) candidates (WF: whole fragment; LH: left half and RH: right half as indicated on Fig. 1A, green colored fragments) were integrated into the dTALE AD domain adjacent portion by fusion PCR (the procedure is shown on Fig. S1), then subcloned into dTALE *Bbv*CI and *Xba*I site to assure that only the dTALE AD domain was replaced and to create the final construct, pDW2177ARS_CIA5. For expression in Chlamydomonas, this dTALE construct was used to transform CW10 (CC849, $mt^{-)}$ cells by the glass bead transformation technique [24]. ARS activity in transformants was assayed as previously described [22].

2.4. Construction of mini-CIA5 and complementation selection

To investigate the function of key CIA5 domains, two conserved regions of the *CIA5* CDS, the putative binding domain, nt 1-330, and the putative activation domain, nt 1282-1671, were fused to generate a recombinant mini-CIA5. This mini-CIA5 was then transformed into *cia5* by glass bead transformation [25] to test whether it could complement the lethal phenotype of *cia5* in very low CO_2 and/or affect downstream gene expression normally regulated by CIA5. Full-length *CIA5* CDS (F-CIA5) also was transformed into *cia5* as a positive control. The primers used to construct mini-CIA5 and F-CIA5 are shown in Table S1. The CIA5 fragments generated by PCR using these primers were then subcloned through the *Nde*I/*Eco*RI multi-cloning site into pGenD_AphVIII plasmid, which is a

modified version of the nuclear expression vector pGenD described by Fischer and Rochaix [26]. For expression in Chlamydomonas, the mini-CIA5 and F-CIA5 were used to transform *cia5* cells by the glass bead transformation technique [24].

To select for complementation of the *cia5* lethal phenotype in very-low CO_2, transformed *cia5* cells were plated on either CO_2 minimal plates or CO_2 minimal with 10 µg/L paromomycin (Par), and the plates were placed into either high CO_2 (aerated with 5% CO_2) growth chamber (plates with Par) or very low CO_2 (50-100 ppm CO_2) chamber (plates without Par). Par^R transformants from the high CO_2 growth plates were transferred to very low CO_2 for complementation screening. Colony-PCR [27] was used to confirm the presence of the transgene in surviving colonies, and spot-tests were applied to confirm the complementation by growth.

2.5. Gene expression analysis

RNA was extracted using an RNeasy Plant Mini Kit (Qiagen, Cat. No. 74904), and the RNA concentration was measured using an ND-2000 Nanodrop spectrophotometer (Nanodrop Technologies). 1-5 µg total RNA was treated with TURBO DNA-Free™ kit (Life Technologies), and cDNA was generated from 0.5 µg -1 µg of treated total RNA (Verso cDNA Synthesis Kit; Thermo Scientific). The resulting cDNA (25-50ng) was used for reverse transcription polymerase chain reaction (RT-PCR) and for quantitative RT-PCR (qRT-PCR). Primer sequences and efficiencies are provided in the supplemental information (Table S1 and Table S3). The qRT-PCR was performed on a CFX Connect™ Real-Time PCR Detection System (Biorad) using a SYBR green two-step quantitative PCR system

(Quanta Biosciences). For quantitative analyses, the *CBLP* (Chlamydomonas G protein beta subunit-like polypeptide) gene was used as an internal control for normalization of qRT-PCR data [17]. The relative transcript abundance in each sample is defined as $\Delta Ct = Ct_{(target\ gene)} - Ct_{(CBLP)}$ to represent the difference between the transcript abundance of genes examined and the transcript abundance of CBLP. After normalization, ΔCt values of each transformant were compared with that of wild type cells, and the relative gene expression is represented by $\Delta\Delta Ct = \Delta Ct_{(transformant)} - \Delta Ct_{(wild\ type)}$ [22].

2.6. Western immunoblot analysis

For western immunoblotting, total protein was obtained as described previously [28]. Proteins were separated by SDS-PAGE on 4-12% polyacrylamide gels. Immunoblotting utilized an anti-FLAG-tag antibody (Santa Cruz Biotechnology; catalog no. sc-51590) and was performed as described in the protocol from Bio-Rad Laboratories (product catalog no. 500-0006).

2.7. Expression of protein fragments in E. coli

A fragment of the *CIA5* gene from *Eag*I (1214) to *Not*I (1930) sites and all other fragments used in this study were sub-cloned into pET30 vector and then transformed into *E. coli*. Cells were IPTG induced at 37°C. Proteins were separated by SDS-PAGE on 8% or 10% polyacrylamide gels, and detected by immunoblotting using an anti-His-tag antibody.

2.8. Chlorophyll measurement

The chlorophyll content was measured as described by Glaesener *et al.* [29] with calculations were based on Porra *et al.* [30].

3. Results

3.1. Identification of the CIA5/CCM1 auto-activation domain using yeast

Yeast two-hybrid screening is a technique used to discover protein-protein interactions by testing for physical interactions (such as binding) between two proteins. The most common screening approach involves the GAL4 yeast two-hybrid assay [31]. Using a yeast two hybrid GAL4 system to detect CIA5/CCM1 interacting components, we found too many false positive yeast colonies to count when the full length *CIA5* cDNA was fused with the cDNA encoding GAL4 DNA binding domain (fragment 1 in Fig. 1A) as bait to screen a cDNA library [20, 32]. We hypothesized that CIA5/CCM1 itself can auto-activate reporter genes when fused with GAL4 DNA binding domain in yeast, so we introduced the full-length *CIA5* fused with GAL4 DNA binding domain in yeast without the prey plasmid, and again found it can activate expression of the reporter genes. Thus, the CIA5/CCM1 protein itself appears to have auto-activation activity, at least in yeast.

Taking advantage of this auto-activation effect in yeast, we were able to narrow down which CIA5/CCM1 regions might act as putative activation motifs. In testing several -partial CIA5/CCM1 fragments (Fig. 1A, fragments 2-28), we found that any CIA5 fragments that included aa 436 to 544 (golden color region shown on Fig. 1A), including fragments 17, 18,

21 or 22, could auto-activate the reporter genes when fused with the GAL4 DNA-binding domain in the absence of the GAL4 activation domain (Fig. 1B and Table S2). This 109-aa (436aa - 544aa) delineated auto-activation region agrees very well with the 130-aa C-terminal region highly conserved between *Volvox carteri* (hereafter, *Volvox*) CCM1 and Chlamydomonas CIA5/CCM1 (aa 428 to 557; i.e., the region of Fig. 1A boxed in red) [33].

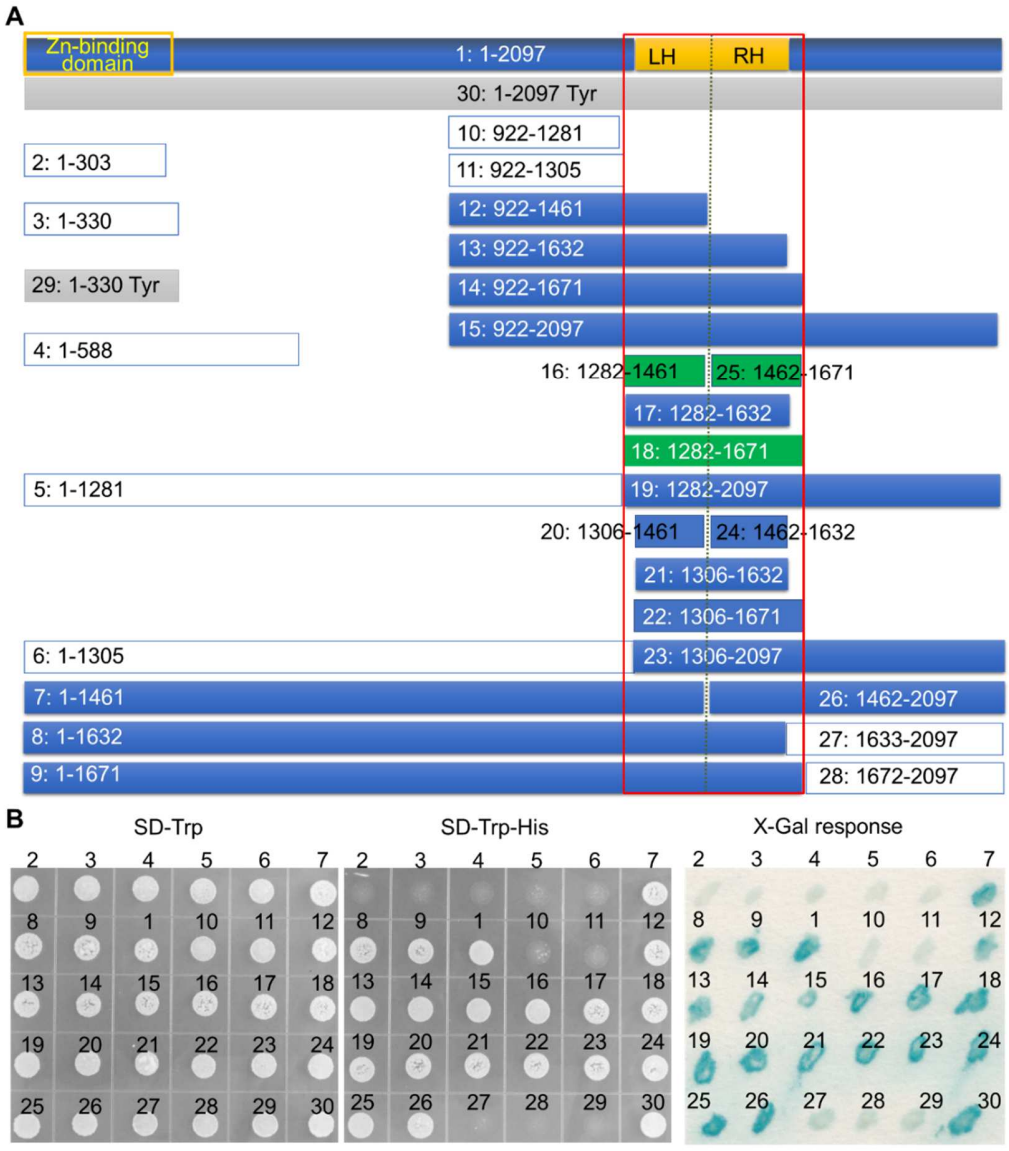

Fig. 1. Testing of *CIA5* fragments as activation domains in the yeast two-hybrid system. A. *CIA5* full coding DNA sequences (CDS) or DNA fragments containing regions of the CDS used for yeast two hybrid experiments. The uppermost bar represents the full CIA5 coding sequence. The position of each fragment in the CIA5 coding sequence is indicated by its position relative to the full coding sequence and by the numbers representing its span of nucleic acid residues in the coding DNA sequence. Fragments colored either blue (■) or green (■) showed auto activation in the yeast two hybrid assay. Fragments lacking color were negative when tested using the two hybrid assay. The golden box (□) indicates the N-terminal Zinc-binding domain and the red box (□) indicates the C-terminal region conserved between Chlamydomonas and *Volvox* and that overlaps with the auto activation region identified using the yeast two hybrid assay, and the grey dashed line (⋮) splits this region into two halves. The golden color region (■)is the region which was identified as promoting auto activation in the two hybrid assay The green colored fragments (16, 18 and 25) were used for replacing dTALE activation domain and designated as LH (16: 1282-1461, left half), WF (18: 1282-1671, whole fragment) and RH (25: 1462-1671, right half) respectively. The grey colored fragments (29 and 30) are those containing a His^{54} to Tyr^{54} point mutation. B. Results of yeast two hybrid experiments using CIA5 CDS and CIA5 CDS fragments, but in the absence of activation-domain partners to identify auto-activation domains. pGBKT7 constructs containing either full length or partial *CIA5* cDNAs (numbered as indicated in Figure 1) were transformed into yeast AH109, and selected using SD dropout Trp (SD-Trp) selective plates. Transformants were then transferred to SD dropout Trp and His (SD-Trp-His) selective plates to screen for possible auto-activation. Any growth on SD-Trp-His medium indicates potential auto-activation candidates. X-gal induction was used as a

secondary screen to confirm auto-activation. Because α-galactosidase is a secreted enzyme, it can be assayed directly using X-α-Gal indicator plates.

We further investigated the CIA5 fragments capable of auto-activation in yeast and discovered that either half of the 109-aa or the 130-aa conserved C-terminus domain (Fig. 1A, fragments 16, 20, 24, & 25) could activate the reporter genes in yeast. Results from experiments using either the SD dropout selective medium assay or an X-gal test (Fig. 1B and Table S2) confirmed that either the 109-aa and 130-aa region or half of these regions behave as true activation domains in yeast. Only the 130-aa region was further studied.

3.2. CIA5 activation domain confirmation in Chlamydomonas

Although the efficiency for activating target gene expression is relatively low, a designed transcription activator-like element (dTALE) system can be used to target and activate expression of the arylsulfatase 1 (ARS1) gene in Chlamydomonas [22, 34]. We reasoned that we could test the activation function of putative activation domains of CIA5 in Chlamydomonas cells by using them to replace the TALE activation domain [23] in this dTALE system.

3.2.1. Expression of dTALEs in Chlamydomonas cells

We used a two-step PCR reaction to create constructs pDW2177ARS_CIA5 containing either the entire 130-aa (aa 428 - 557) putative activation domain (pDW2177ARS_CIA5_WF)

or each half of this domain (either pDW2177ARS_CIA5_LH or pDW2177ARS_CIA5_RH) (Fig. S1). We then transformed wild-type Chlamydomonas strain CW10 (CC849) with these dTALE constructs carrying an antibiotic gene (Ble), and selected transformants based on resistance to zeocin (10 μg/mL). The original dTALE construct, pDW2177ARS [22], and a modified dTALE lacking any activation domain, pDW2177ARS-AD, were used as positive and negative controls, respectively (Fig. S2). We confirmed the insertion of intact dTALE constructs into the Chlamydomonas genome in about 20-40% of the 192 zeocin resistant transformants, screened for each of the five constructs using colony PCR (Fig. S3).

3.2.2. dTALEs containing CIA5 activation domains induced expression of ARS1 in Chlamydomonas

A colorimetric assay was performed to screen for ARS activity in the transgenic cells grown under sulfur-replete conditions. This assay is capable of detecting activation of *ARS1* expression in the absence of sulfur stress – a condition under which the ARS1 and ARS2 genes are typically fully repressed. Of the 192 transformants screened for the positive control (pDW2177ARS) and each of the three CIA5-fragment-containing pDW2177ARS-derived constructs (pDW2177ARS_CIA5_WF, pDW2177ARS_CIA5_LH or pDW2177ARS_CIA5_RH), ~5-20% of each transformation showed cleavage of the XSO_4 (5-bromo-4-chloro-3-indolyl-β-D-SO_4) substrate and demonstrated concomitant color changes in sulfur-replete culture medium (representative data in Fig. 2A; not all data shown). All transformants testing positive for ARS activity contained an intact dTALE construct as determined by PCR amplification of the gene. None of the transformant colonies lacking a

PCR-detectable intact dTALE construct showed detectable color change under the same growth conditions. Of 192 negative control transformants (i.e., those transformed with pDW2177ARS-AD which lacks an activation domain), 42 lines (21.9% of all transformants screened) contained a PCR-detected intact dTALE. However, none of these 42 lines showed detectable ARS activity (representative data for four transformants (-AD1, -AD2, -AD3 and -AD4) are provided in Fig. 2A; not all data shown).

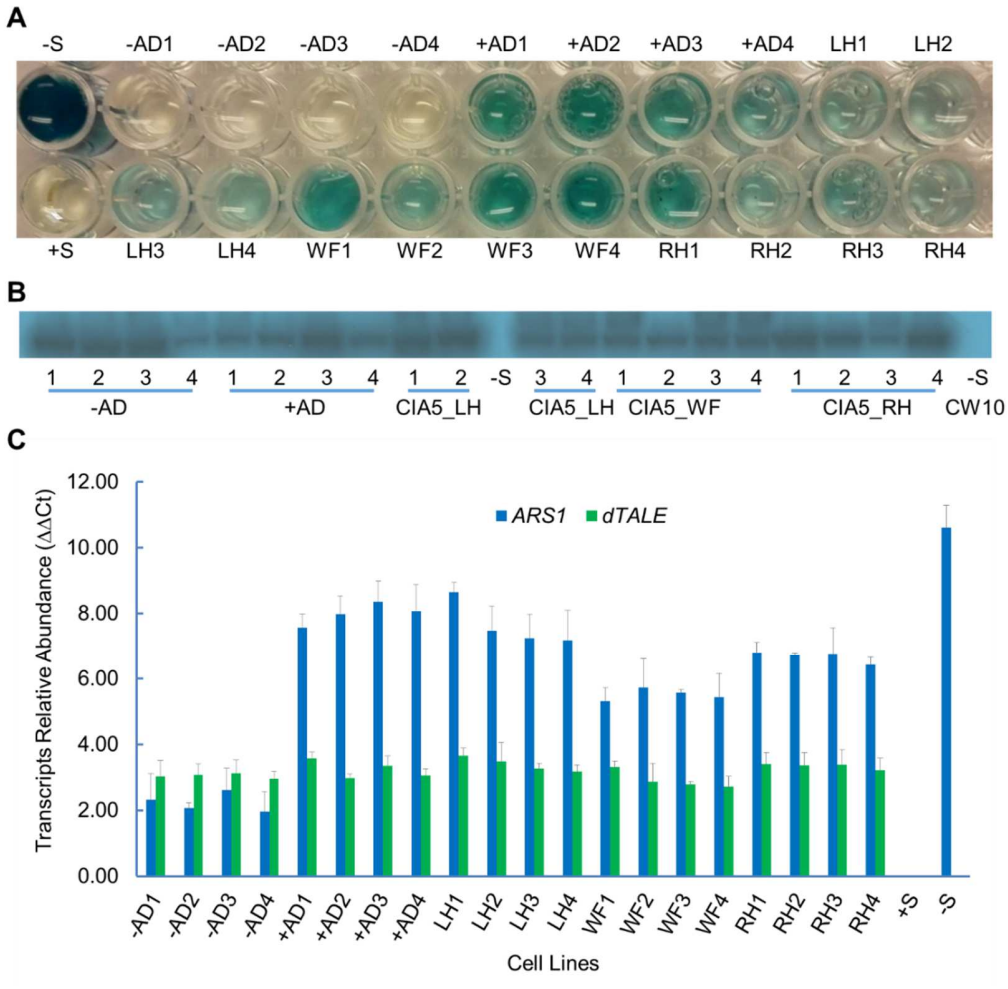

Fig. 2. Representative examples of colorimetric screens for ARS activity induced by dTALEs. CW10 cells grown either with (+S) or without (-S) sulfur serve as negative and

positive controls, respectively. Other transformants were all grown with sufficient sulfur for 60 hours, then incubated with XSO_4. The blue-green color indicates ARS activity. Microtiter wells -AD1 to 4 contain cells transformed with the pDW2177ARS-AD construct lacking an activation domain; wells +AD1 to 4 contain cells transformed with construct pDW2177ARS that contains the native TALE activation domain; wells LH1 to 4 contain cells transformed with pDW2177ARS_CIA5_LH that contains the left half of the CIA5 activation domain; wells WF1 to 4 contain cells transformed with pDW2177ARS_CIA5_WF that contains the entire CIA5 activation domain; wells RH1 to 4 contain cells transformed with pDW2177ARS_CIA5_RH that contains the right half of the CIA5 activation domain. B. Western immunoblots of Chlamydomonas cells transformed with various pDW2177ARS-derived constructs showing the relative abundance of the dTALE proteins. Anti-Flag-tag antibody was used as described in Material and Methods. Cells were grown under sulfur replete conditions for 60 hours. Non-transformed CW10 cells grown without sulfur (-S) were used as a control (middle and last columns). C. qRT-PCR analysis of *ARS1* target gene and dTALE gene transcript abundances in cells transformed with constructs containing various activation domains. Columns 1-20: *ARS1* and *dTALE* transcript abundances (Relative log2 Fold Change) for selected transformed lines that contained intact dTALE-ARS1 constructs (with and without various activation domains) including those (columns 5-20) that tested positive for ARS activity. Transformants containing constructs lacking an inserted activation domain (-AD 1-4) showed only basal level *ARS* expression. The *ARS* transcript abundance is much higher in those with an inserted activation domain, but not as high as the CW10 sulfur starvation-induced control (-S, last column). $\Delta\Delta Ct = \Delta Ct_{(transformant)} - \Delta Ct_{(WT)} = (Ct_{(target\ gene\_transformant)} - Ct_{(CBLP\_transformant)}) - (Ct_{(target\ gene\_WT)} - Ct_{(CBLP\_WT)})$, where ΔCt equals the

difference between target transcript abundance and the transcript abundance of the *CBLP* gene, the internal control gene. Three technical replicates were performed for each transformant and the error bar represents the standard error. Transformed lines were grown under sulfur replete conditions, while CW10 cells were grown under either sulfur-replete or sulfur-deficient conditions, as indicated, for 60 hours. Numbers underneath each panel indicate individual transformant lines.

Successful expression of dTALE constructs at the transcript level was confirmed using RT-PCR (Fig. S4). We tested 4 candidate transformant lines for each of the 5 pDW2177ARS-derived constructs, using primers specific for the 5' region of the dTALE constructs. All showed detectable expression of dTALE-ARS1 transcripts. We also used western immunoblot analysis to detect dTALE expression at the protein level. Of all tested transformant lines exhibiting RT-PCR-detectable expression of dTALEs, protein expression was confirmed using anti-FLAG tag antibody (Fig. 2B). The expression of dTALE also was detected in pDW2177ARS-AD transformants lines (lacking an activation domain) at both the transcript and protein levels (Figs. 2B & S4).

Activation of the target gene, *ARS1*, was also demonstrated at the transcript level by RT-PCR with primers specific for the *ARS1* 3'UTR. When grown under sulfur starvation, elevated expression of *ARS1* was observed in wild type cells (Fig. S4). dTALE-induced production of *ARS1* transcripts and positive colorimetric assays in cells grown under sulfur-replete conditions were observed in all tested lines that contained intact pDW2177ARS-derived dTALE construct and showed colorimetric ARS activity (positive control and three CIA5-fragment-containing constructs). In contrast, pDW2177ARS-AD lines (i.e., lines

transformed with dTALE construct lacking activation domain) displayed an intact pDW2177-ARS1 dTALE construct but no detectable colorimetric ARS activity (Figs. 2A & S4).

3.2.3. Each half of the CIA5 activation domain can function as an independent activation domain

All three dTALE constructs containing the putative CIA5 activation domain or its left or right partial domains (i.e., pDW2177ARS_CIA5_WF, pDW2177ARS_CIA5_LH, and pDW2177ARS_CIA5_RH, respectively) could activate expression of the target gene, ARS1, under sulfur-replete conditions (Fig. S4). To gain a more quantitative appraisal of gene transcription enhancement supported by the p2177ARS_CIA5-based dTALE constructs, we used RNA from the same transformed lines employed above to conduct qRT-PCR analyses. The abundance of *ARS1* transcripts in all examined lines from the positive control (i.e., CW10 -S) and the three CIA5-activation domain-containing constructs (i.e., dTALE containing an activation domain) (Fig. 2C) were all positively correlated to the levels of ARS activity observed in the *in vivo* measurements of ARS activity present in cultures of transformed cells (Fig. 2A). Transformants lacking an activation domain (i.e., pDW2177ARS-AD) showed only lower, basal level ARS expression, even though the dTALE abundance was similar to those in all other transformants (Fig. 2C). The relationship between qRT-PCR based *ARS1* transcript abundance and colorimetric ARS activity assays performed on transformants expressing pDW2177ARS-based dTALEs in sulfur-replete medium showed that colorimetric ARS activity was detectable in only those selected lines

with substantial transcript abundance. Interestingly, the levels of *ARS* gene transcripts in transformants containing constructs encoding the left or right fragments of the CIA5 activation domain were somewhat higher than in the constructs carrying the intact activation domain, but this did not translate to markedly increased responses in the ARS colorimetric analyses (Fig. 2A).

3.3. The CIA5 activation domain is also responsible for abnormal migration of CIA5 during SDS-PAGE

Intact CIA5 always shows abnormal migration during SDS-PAGE (i.e, an apparent molecular size of ~95 kD vs. the expected 72 kD), whether obtained from Chlamydomonas cell extracts and detected with anti-CIA5 antibody [11, 19] , or from extracts of *E. coli* overexpressing His-tagged CIA5 and detected with anti-His antibody (Fig. S5). To investigate which region of CIA5 is responsible for the abnormal SDS-PAGE migration, we expressed in *E. coli* different constructs bearing either full length or partial CIA5 coding regions (all containing C-terminal extensions encoding a 6xHis-tag) (Fig. 3) and then detected their expression using anti-His-tag antibody to probe blots of proteins separated by SDS-PAGE (Fig. S5).

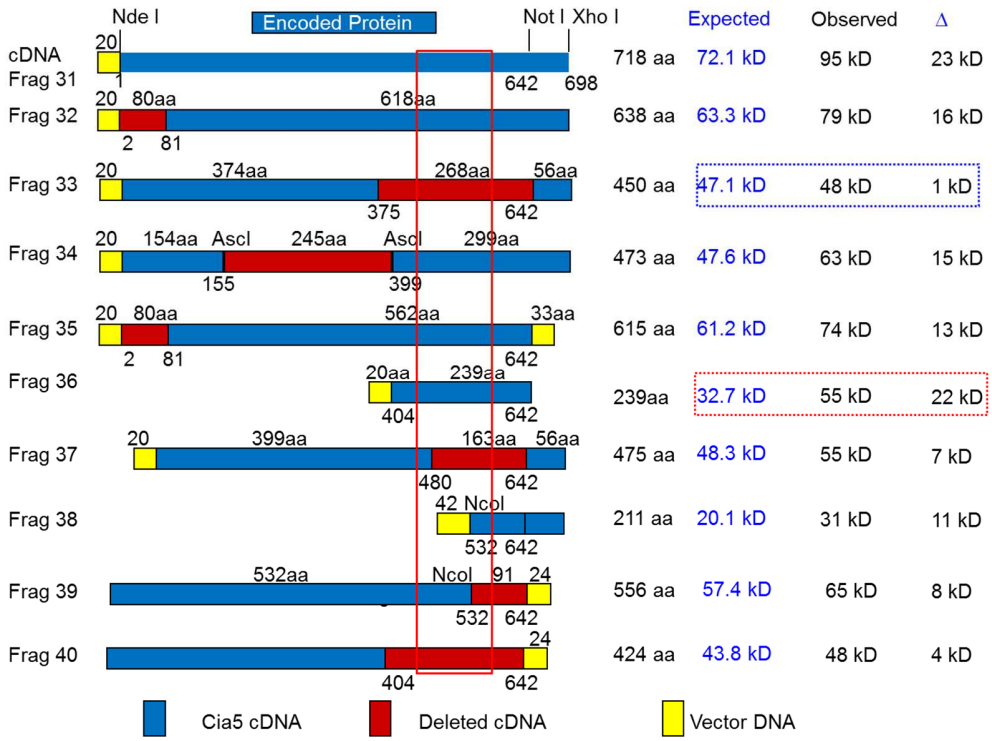

Fig. 3. Migration of CIA5 and CIA5 fragments during SDS-PAGE. CIA5 fragments produced in *E. coli* were used for testing the effect of partial deletions on protein migration during SDS-PAGE. Beside each construct is indicated the predicted mass of the CIA5 fragment, the mass of the fragment deduced from migration of protein standards during SDS-PAGE and the difference between the two - a measure of migration aberrancy. Lengths (in amino acid numbers) of CIA5 polypeptides (blue boxes) and deleted regions (red boxes) are provided above each cartoon. Starting and finishing points (in amino acid position) for each deletion are provided below each cartoon. Vector sequences are shown as yellow boxes. The open red box (□) indicates the approximately 130-aa activation domain region of CIA5.

As shown both in Fig. 3 and Fig. S5, a construct containing the full-length *CIA5* cDNA (Frag 31, Fig. 3 and Fig. S5) produced a protein migrating as a ~95 kD protein instead of the

expected 72.1 kD (i.e., an approximate 23 kD difference between observed and predicted molecular mass). Indeed, any partial cDNA containing the coding region for amino acids from residue 402 to residue 642 [or the *Asc*I-*Not*I region (aa 399-642)] showed aberrant migration rates during SDS-PAGE (i.e., Fragments 32, 34, 35, 36, 37, 38, 39, and Frag 40 in Fig. 3 and Fig. S5). Those constructs containing Frag 36 (aa 404 to 642) show the greatest abnormal SDS-PAGE migration (about 22 kD difference from the expected). The constructs containing only a small part of this region (Frag 37, 38, 39 and Frag 40 in Fig. 3 and Fig. S5) show less abnormal migration, and those including no part of this region (e.g., Frag 33 in Fig. 3 and Fig. S5) exhibit no abnormal migration. This apparent size discrepancy is observed both with CIA5 isolated from Chlamydomonas and with CIA5 produced from *E. coli* and, thus, is not likely due to post-translational modification of the protein. In particular, the aberrant migration also does not result from post-translational SUMOylation of CIA5 because the protein still ran aberrantly and could still complement the *cia5* mutant when every potential CIA5 SUMOylation site was mutated [35].

The region apparently responsible for abnormal SDS-PAGE migration (aa 399 - 642) overlaps extensively with the 130-aa C-terminal CIA5/CCM1 domain conserved between Chlamydomonas and *Volvox* that we demonstrated above to behave as an activation domain in both yeast and Chlamydomonas. The aa sequence of this region contains an abundance of acidic aa residues (E and D), which suggests that it may function as an acidic activation domain [23].

3.4. The activation domain is indispensable for complementation of the cia5 mutant

A number of restriction enzyme-based deletions in the *CIA5* gene were created and tested for their ability to complement the *cia5* mutant. The 130-aa region within the C-terminus of CIA5 (and conserved between Chlamydomonas and *Volvox*) that we confirmed to act as an activation domain in yeast and Chlamydomonas, also appears essential for complementation of the *cia5* mutant. In all cases, constructs were designed (and confirmed by DNA sequencing) to ensure that a proper reading frame for the coding sequence was created. Either removal of the entire 3'-UTR of CIA5 (Figs. 4 & S6, fragment 1) or elimination of DNA downstream of the *NotI* restriction site [i.e., the region encoding aa 642 - 698 (Figs. 4 & S6, fragment 2)] shows no effect on the ability of the resulting constructs to complement the *cia5* mutant. Deletion of the large *Asc*I restriction fragment (encoding aa 153 - 399) (Figs. 4 & S6, fragment 3) or deletion of a fragment encoding aa 261 - 420 (Figs. 4 & S6, fragment 7) from the *CIA5* WT gDNA does not affect the ability of this DNA molecule to complement the *cia5* mutant, but deletion of the larger *Xmn*I-*Not*I restriction fragment encoding aa 192 - 642 (Figs. 4 & S6, fragment 4), which contains the C-terminal conserved domain, prevents complementation.

A

B

Summary of *CIA5* deletions evaluated:	Effect on *cia5* (H54Y) complementation:
1. *CIA5* gDNA Δ 3'-UTR	1. None
2. *CIA5* gDNA Δ *Not*I aa 643-698)	2. None
3. *CIA5* gDNA Δ *Asc*I (aa ~150-400)	3. Minimal
4. *CIA5* gDNA Δ *Xmn*I-*Not*I (aa ~193-643)	4. Complete
5. *CIA5* gDNA Δ aa 430-550	5. Severe
6. *CIA5* gDNA Δ aa 430-487	6. Severe
7. *CIA5* gDNA Δ aa 262-421	7. None
8. *CIA5* WT gDNA	8. None

Fig. 4. Determination of the regions of the *CIA5* gene or cDNA needed for successful complementation of the *cia5* mutant. A. Restriction enzyme map of the CIA5 cDNA showing cleavage sites for restriction enzymes used to create various cDNA (or gDNA) deletion mutants used in complementation assays. Exons E1 through E6 comprising the CIA5 cDNA are depicted in the pink bar below the restriction map. The location of the coding region for the CIA5 activation domain is shown as a blue bar. B. Summary of results of complementation experiments with intact and deleted *CIA5* constructs.

This large coding region overlaps with the *Eag*I-*Not*I region responsible for the aberrant SDS-PAGE migration (see above) and the conserved 130-aa putative activation domain associated with gene activation in yeast and Chlamydomonas. Deletion of the *Eag*I-*Not*I restriction fragment (encoding aa 403 - 642) also eliminates CIA5 function. Deletion of the approximate 130-aa region aa 430 - 550 (Figs. 4 & S6, fragment 5) also eliminates the *CIA5* function. Even deletion of only nucleotides 1288 to 1458 (encoding aa 430 - 487), approximately the first half of the 130-aa C-terminal putative activation region conserved between Chlamydomonas and *Volvox*, also eliminates the ability of the resulting CIA5 fragment to complement *cia5* (Figs. 4 & S6, fragment 6).

3.5. Recombinant mini-CIA5 construct can complement cia5

Both mini-CIA5 and full length *CIA5* CDS were subcloned into pGenD_AphVIII vector through *Nde*I/*Eco*RI (Fig. S7), then transformed into *cia5* by glass bead transformation [25]. Cells were plated on either CO_2 minimal medium or CO_2 minimal medium with 10 μg/mL

paromomycin (Par), the plates were placed into either high CO_2 (aerated with 5% CO_2) growth chamber (plates with Par) or very low CO_2 (50-100 ppm CO_2) chamber (plates without Par). The Par^R transformants from the high CO_2 growth plates were transferred to very low CO_2 for complementation screening. The majority of Par^R transformant colonies obtained from high CO_2 either died or were unable to continue growth after transfer to very low CO_2 for about one month. After 4-5 weeks selection under very low CO_2, we obtained 5 Par^R transformants from the mini-CIA5 (hereafter named mini-CIA5) construct and 11 Par^R transformants from the full length *CIA5* CDS construct (thereafter named F-CIA5). Colony-PCR was used to confirm the presence of the transgene, and spot-tests were applied to confirm the complementation by growth. Primer pair P1/P6 was used for colony PCR [27], and could only amplify a 435-bp genomic DNA fragment from the control lines (CW10, *ad1* and *cia5*), but could amplify both a 330-bp CDS fragment and the 435-bp genomic fragment with DNA from transformants complemented with either mini-CIA5 or F-CIA5 (Fig. 5A). Sequencing of both these bands obtained using colony PCR of DNA from the complemented lines revealed that the genomic DNA band still retained the original CIA5 gene point mutation, confirming that the complemented lines were not revertants. Unlike the *cia5* mutant itself, *cia5* transformants complemented with either mini-CIA5 or F-CIA5 were able to grow under very low CO_2, just like the control strains *ad1* and CW10 (Fig. 5B). Interestingly, Par^R was lost (presumably silenced) in all the complemented *cia5* transformants after long selection under very low CO_2 conditions in the absence of Par.

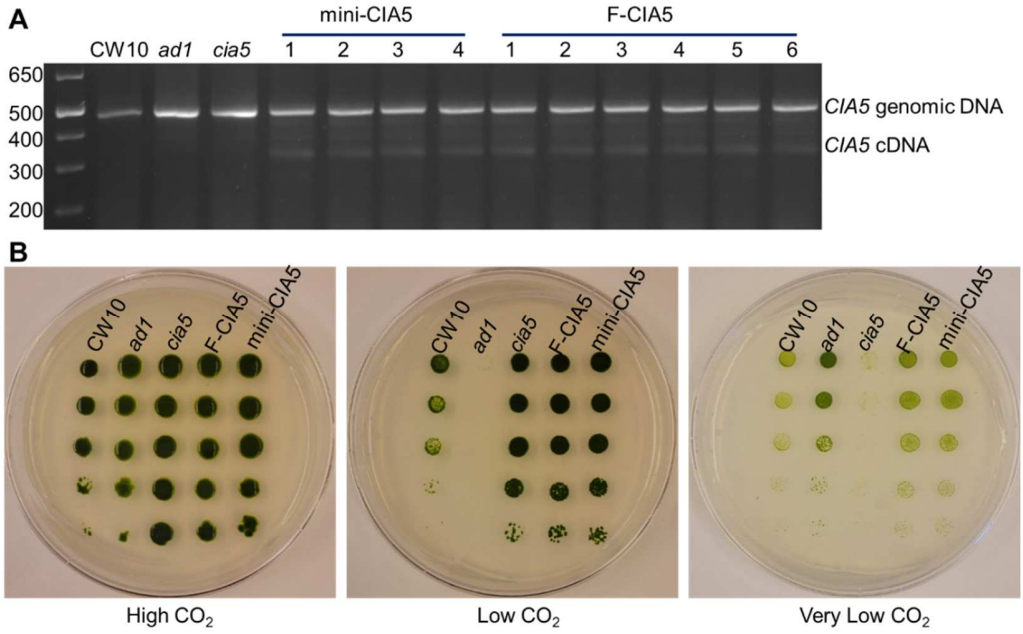

Fig. 5. Comparison of growth of *cia5* mutants transformed with a full-length *CIA5* gene or the mini-*CIA5* gene construct. A. Colony PCR assessing the presence or absence of *CIA5* CDS in transformants. The primer pair P1/P6 (Table S1) which can amplify either a 330 bp fragment with *CIA5* CDS as the template or a 435 bp fragment with *CIA5* genomic DNA as the template was used to perform colony PCR. The transformants that carried either *CIA5* full CDS (F-CIA5) or partial CDS (mini-CIA5) should have both the 330 bp and 435 bp PCR products, while the control lines CW10, *ad1* and *cia5* only contain genomic DNA template, so should amplify only the single 435-bp PCR product. B. Spot test for growth of control lines (CW10, *ad1* and *cia5*) and *cia5* transformants (F-CIA5 and mini-CIA5) on minimal medium in high (5%) CO_2, low (air-level; ~400 ppm) CO_2 or very low (~100ppm) CO_2. Cells grown in liquid medium in high CO_2 to logarithmic phase were diluted to similar concentrations, spotted (5 µl) on plates in a five-fold dilution series and incubated for 9 days under three different CO_2 concentrations.

3.6. Expression of CO_2-responsive genes in mini-CIA5 transformants

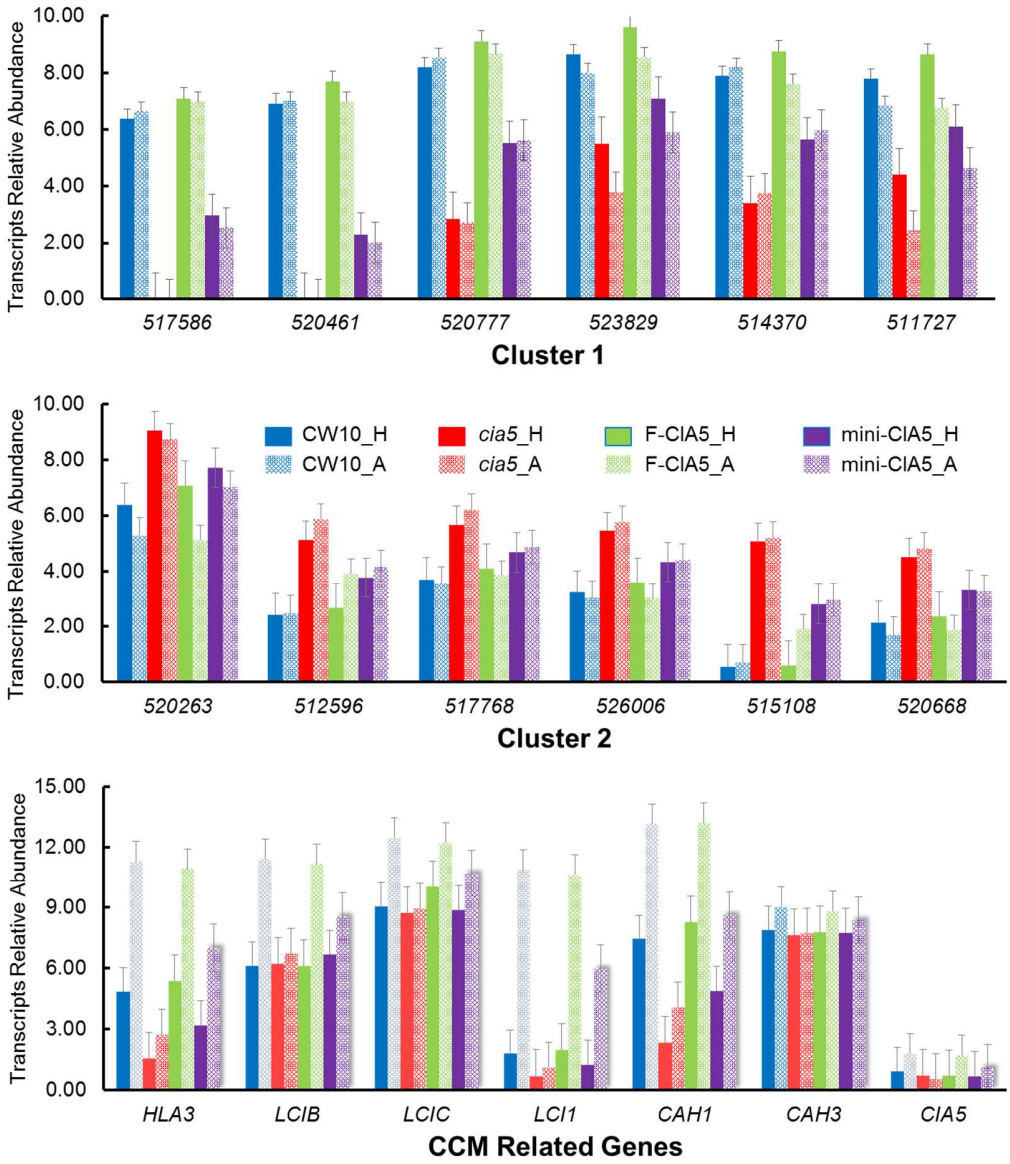

Fig. 6. qRT-PCR analysis of expression of CO_2-responsive genes from different transcriptome categories. Transcript relative abundance (ΔΔCt) are depicted for CW10 (blue color), *cia5* (red color) and *cia5* transformants complemented with either *CIA5* full length CDS (F-CIA5) (green color) or *CIA5* partial CDS (mini-CIA5) (purple color) under either

high CO_2 (H) (solid fill) or air-level CO_2 (A) (pattern fill) conditions. The names or assigned numbers for candidate genes from each category are listed in Table S3. Three replicates were performed for each strain. Error bars represent the standard error.

Although the mini-CIA5 construct can complement *cia5*, whether it functions fully or partially with regards to all types of CIA5-regulated genes is in question (Fang et al. 2012). Based on transcriptome data, transcript abundance for over 1,000 genes appears to be affected mainly by CIA5 but only minimally by CO_2 in "CIA5 clusters" [17]. Among genes from typical "CIA5 clusters", cluster 1 genes show high expression in WT but low expression in *cia5*, and cluster 2 genes show the opposite expression pattern. Several genes from both clusters (Cluster 1 and Cluster 2), as well as a few representative, well-characterized CCM-related genes, were selected to test their expression in the mini-CIA5-complemented *cia5* lines to compare functions of mini-CIA5 vs full length CIA5. Ten candidates from each of these 3 categories were chosen for initial screening (detailed information, such as gene names or transcript IDs and primers used for RT-PCR and qRT-PCR, are included in Table S3). All primers were tested by RT-PCR for 30 cycles in CW10 or *cia5* (primers from cluster 2), and the amplification efficiency for each primer pair is shown in Fig. S8A. The primers for candidate genes with good amplification efficiency were used to perform both RT-PCR and qRT-PCR in cell lines, including CW10, *cia5*, *cia5* transformants complemented with mini-CIA5, and *cia5* transformants complemented with F-CIA5, under high CO_2 (H) and air level CO_2 (A) conditions.

F-CIA5 transformants complemented with CIA5 full length CDS enable the complemented transformants to function very similarly to the CW10 WT strain with regard

to expression patterns and amplitudes of all candidate genes investigated (see F-CIA5 in Fig. S8B, C and D, and Fig. 6A, B and C). On the other hand, mini-CIA5 transformants complemented with mini-CIA5 carrying only a partial CIA5 CDS (nt 1-330 plus nt 1282-1671), although clearly complementing *cia5* and showing similar expression patterns, did not function exactly the same as either CW10 or F-CIA5-complemented lines, with regard to expression amplitude for any of the candidate genes for which CIA5 functions as either an apparent activator (cluster 1 plus some CCM-related genes) or an apparent repressor (cluster 2). When complemented with mini-CIA5, the expression amplitude of each of the candidate genes behaved as intermediate between *cia5* and CW10 (see mini-CIA5 in Fig. S8B, C and D, and Fig. 6A, B and C). These results demonstrate that, although mini-CIA5 clearly can phenotypically complement *cia5* and enable the transformants to grow under very low CO_2, mini-CIA5 apparently did not fully activate or fully repress downstream gene expression to the same extent as the full length CIA5, and mini-CIA5 also did not enable the same impact amplitude as CIA5 did when both CIA5 and CO_2 affected expression of CCM related genes (see CCM related genes in Fig. S8B, C and D, and Fig. 6A, B and C).

3.7. Analysis of growth of mini-CIA5 transformants in liquid culture

We compared the growth rate and chlorophyll content of the complemented *cia5* transformants (F-CIA5 and mini-CIA5) with those of CW10, *ad1* and *cia5* under high CO_2, air-level CO_2 and very low CO_2 conditions. Growth experiments showed patterns of photoautotrophic growth for *cia5* transformants consistent with those observed in spot tests (Fig. 5A). Actively growing, 2-d-air-acclimated cells were inoculated into liquid minimal

medium with similar starting cell densities (1×10^5 cells mL^{-1}), grown with aeration, and the cell densities measured by OD_{750} daily at the same time of day for 10 d. Not much difference was observed among all strains grown under high CO_2 (Fig. 7A), except the maximum density of mini-CIA5 transformants from day 8 forward was lower than the others. Also, in air-level CO_2, the cell densities for CW10, F-CIA5, mini-CIA5 and *cia5* were all lower than those in high CO_2 but almost uniformly much higher than that of *ad1* (Fig. 7B), except that the mini-CIA5 complemented line again was slightly lower than the others from day 10 forward. However, in very low CO_2, all strains exhibited a markedly decreased growth rate and a much lower final cell density than in high or air-level CO_2, with CW10, *ad1* and F-CIA5 showing similarly higher growth rates and final cell densities than *cia5*, while mini-CIA5 exhibited a growth rate and final cell density intermediate between CW10 (or F-CIA5 or *ad1*) and *cia5* (Fig. 7C). Chlorophyll concentration also was measured in these cultures during the same time course, with the growth curves based on chlorophyll exhibiting a pattern similar to those for OD_{750}-based cell density (Fig. 7 D-F).

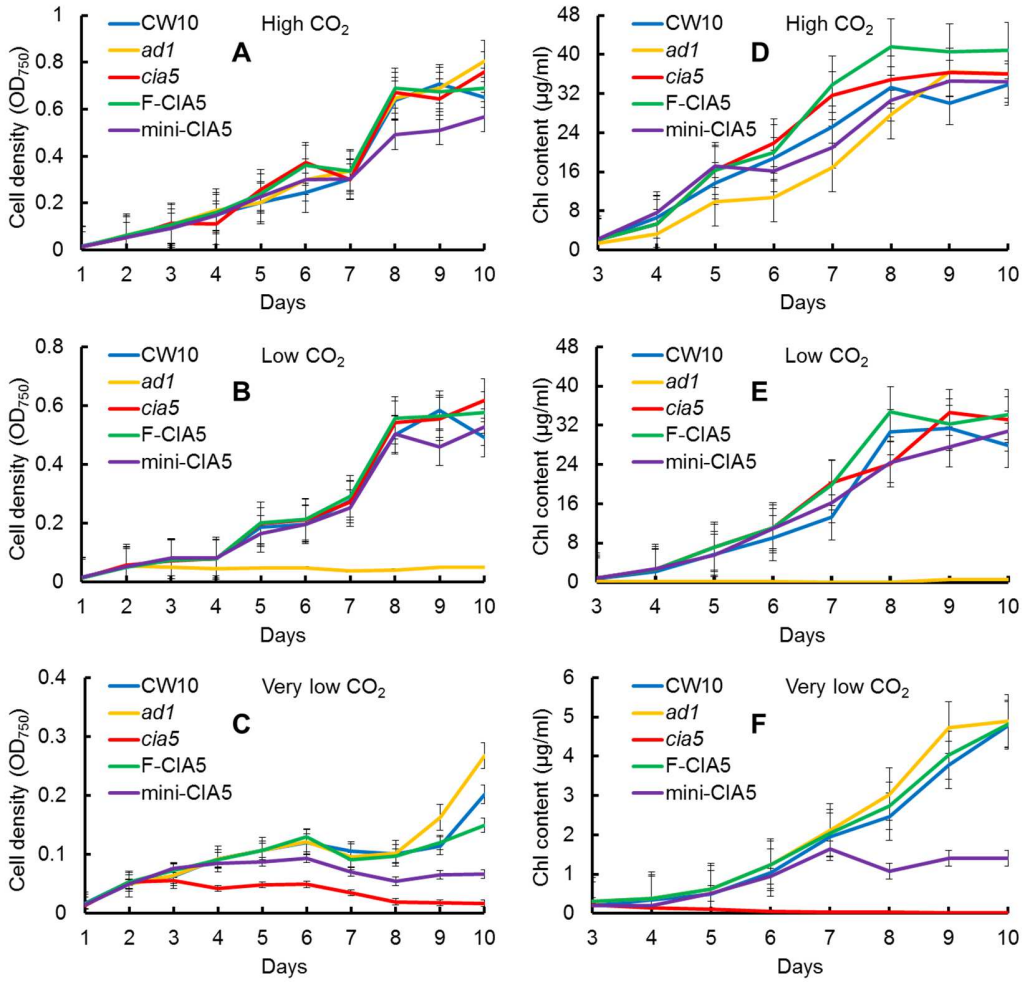

Fig. 7. Cell density and total chlorophyll (Chl) concentration under three CO_2 conditions. Cell density was measured and monitored as the absorbance at 750 nm using clear, flat-bottom 96-well plates. Chlorophyll concentrations were measured by monitoring the absorbance at 750 nm, 663.6 nm and 646.6 nm using clear, flat-bottom 96-well plates, and employing the flow-chart and calculations based on Porra et al. [30]. Three replicates were performed for each measurement.

4. Discussion

As a master regulator of the CCM in Chlamydomonas, CIA5 may function alone or, more likely, work with other factors to control downstream gene expression. *CIA5* was identified independently by two research groups [12, 13], but little progress has been made in understanding how CIA5 regulates such a large network of genes [17]. The regulation of thousands of genes, including induction or up-regulation of almost all limiting-CO_2 inducible genes (including most putative Ci transporters and some carbonic anhydrases) is impaired in the *cia5* mutant [10, 11, 17].

CIA5/CCM1 contains two N-terminal zinc-binding domains. If one of these zinc-binding domains is mutated, H54Y as in the *cia5* mutants [12, 13], CIA5 activity is lost and the regulation of most CO_2-responsive genes is disrupted. Aside from this role as the regulator of the CCM and other low CO_2 acclimation responses, the details of CIA5 function remain largely undefined. Transcriptome analyses of cells shifted from high to low CO_2 conditions identified a massive impact of CIA5/CCM1 and CO_2 on the transcription of several thousand genes and revealed an array of gene clusters with distinctive expression patterns [17, 18]. These observations provide important insights into the regulatory interaction between CIA5 and CO_2 and the knowledge that individual gene clusters responded primarily to CO_2-regulated changes in CIA5 activity [17].

The yeast two-hybrid system helped us identified a 109aa region (436aa - 544aa) of CIA5/CCM1 that corresponds to a 130-aa region (428aa - 557aa) highly conserved between Chlamydomonas CIA5 and *Volvox* CCM1 - a region that also exhibits auto-activation in yeast. Replacement of the normal TALE activation domain in a dTALE with this 130-aa putative activation domain demonstrated that this region also behaves as an activation domain in Chlamydomonas, suggesting it also may function as an activation domain in

CIA5. Deletion of the *Xmn*I-*Not*I region, which contains the 130-aa C-terminal conserved, putative activation domain, totally eliminates the ability of CIA5 to complement the *cia5* mutant, suggesting that this region has an essential function in CIA5.

The 130-aa, conserved C-terminal putative activation domain contains an abundance of acidic amino acid residues, and is included within the *Asc*I and *Not*I region (aa 399-642) responsible for abnormal migration of CIA5 during SDS-PAGE. Both observations are consistent with the identified 130-aa conserved C-terminal region acting as an acidic activation domain.

This 130-aa, putative activation region may represent two independent activation domains (aa 428 – 487, LH; aa 488 – 557, RH), since each half of the 130-aa domain could function in auto-activation in yeast as well as in the dTALE system to activate gene expression in Chlamydomonas. Both RT-PCR and qRT-PCR indicated that the *ARS*1 expression in transformants with either half (LH or RH) of the 130-aa AD domain is somewhat higher than in transformants containing the entire (WF) AD (Figs. 2C and S4). Thus, it could be argued that there is a repressor domain within this 130-aa region and this putative repressor domain is destroyed when the 130-aa region is split into two halves. However, deletion of only aa 430-487, approximately the first half of the 130-aa, C-terminal, conserved domain, totally eliminates the ability of CIA5 to complement the *cia5* mutant. This observation argues that the second putative activation domain within this 130 aa region is not sufficient by itself to allow CIA5 function.

The present work represents the first demonstration that a conserved, acidic domain from the CCM master regulator, CIA5, can function as an activation domain both in Chlamydomonas and in yeast. This conserved acidic activation domain also appears

indispensable for CIA5 function, as judged from results of complementation experiments with the *cia5* mutant. Together, these observations provide strong suggestive evidence that CIA5 functions, at least in part, through gene-direct activation of gene expression. Whether CO_2-responsive genes are specifically targeted for activation by CIA5 alone or by collaboration of CIA5 with specific transcription factors remains a prime question to be answered in future studies.

Increased mobility during SDS-PAGE migration has been reported to occur when amino acid residues are substituted that decrease the net negative charge on human superoxide dismutase [36]. These studies demonstrated that aa substitutions such as H80R, G85R, D90A, G93R, E100G, E100K, D101G, and D101N can increase migration (by ~2 - 3 kD) during SDS-PAGE by promoting the binding of three to four additional SDS molecules, without significantly altering the secondary structure, while only one substitution, G93D, decreases migration (by ~2 - 3 kD) [36]. A slight discrepancy between the theoretical MW and the MW observed during SDS-PAGE is not uncommon, incomplete unfolding might influence electrophoretic mobility, also, the observed MW of a protein might differ from the theoretical value when the protein binds more or less SDS than it should on average due to amino acid composition, thus appearing to be larger or smaller than expected. It was reported that basic proteins bind more SDS and acidic proteins bind lese detergent than the average [37]. The pI for CIA5 aa 1 - 698 is 5.58, while for the 130-aa conserved C-terminal region is 4.14 (http://web.expasy.org/compute_pi/). Thus, one might reason that it is possible that high density of acidic residues such as the CIA5 130-aa AD region should decrease SDS binding and, therefore, increase migration of CIA5 during SDS-PAGE - just the opposite of our experimental observations. Thus, the cause for abnormal migration of CIA5 in SDS-PAGE

remains enigmatic and will rely on a more detailed future analysis of CIA5 using appropriate biophysical techniques and/or systematic introductions of amino acid changes to determine which amino acid sequences are key to allowing such unusual migration behavior.

The mini-CIA5 construct, which carries only a partial *CIA5* CDS (nt 1-330 plus nt 1282-1671) was transformed into *cia5* to learn more about the domains of CIA5 required for regulation of CO_2-responsive genes. Based on selection of transformants in very-low CO_2, mini-CIA5 appeared able to complement the *cia5* lethal phenotype in very low CO_2. Both spot tests (Fig. 5B) and liquid growth rate experiments (Fig. 7) corroborated the observation that mini-CIA5 transformants, which contained only two small parts of the CIA5 CDS, were able to complement *cia5* and enable growth under very low CO_2. All CCM-related candidate genes investigated here by either RT-PCR (Fig. S8) or qRT-PCR (Fig. 6) responded to mini-CIA5 with similar expression patterns as they did to F-CIA5, including clear CO_2-regulated expression patterns for those genes normally regulated by both CIA5 and CO_2. However, mini-CIA5 complemented transformants exhibited an apparently intermediate response between CW10 and *cia5*, since mini-CIA5 apparently did not fully activate or fully repress downstream gene expression, and also exhibited intermediate growth phenotypes in very low CO_2 liquid culture. These observations together demonstrate that the conserved N-terminal Zn-binding domain (nt 1-330 or aa 1-110), combined with the conserved C-terminal activation domain (nt 1282-1671 or aa 428-557) are sufficient to provide CIA5 function, including responsiveness to limiting CO_2 signal, enabling mini-CIA5 to complement the *cia5* mutant phenotype, and restoring the growth under very low CO_2. However, we can not rule out the possibility that other CIA5 sequences absent from this mini-CIA5 also may be important for a fully functional CIA5 *in vivo*.

Author Contributions

B.C., K.L, T.P., D.P.W. and M.H.S. conceived this project and designed all experiments. B.C., K.L. and T.P. performed experiments. D.D. and Y.W. performed initial yeast two-hybrid cDNA library screenings, and K.H. made the original observation that CIA5 lacking the large *AscI* fragment could still complement the *cia5* mutant. B.C., D.P.W. and M.H.S. analyzed data and wrote the paper.

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Supplemental Data

The following supplemental materials are available.

Supplemental Table S1. A summary of all the primers used in this work.

Supplemental Table S2. *CIA5* Fragments used for yeast two-hybrid auto-activation identification, and the summary for the results from yeast two-hybrid and X-gal screening.

Supplemental Figure S1. Schematic of a two-step PCR (fusion PCR) approach for synthesis and insertion of the *CIA5* activation domain (AD) into pDW2177ARS constructs.

Supplemental Figure S2. dTALE constructs used for testing CIA5 putative activation domains in Chlamydomonas cells.

Supplemental Figure S3. Representative transformants containing intact dTALE constructs as determined by colony PCR assay.

Supplemental Figure S4. RT-PCR analysis of *ARS1* and *dTALE* transgene transcription in Chlamydomonas cells transformed with various pDW2177ARS-derived constructs.

Supplemental Figure S5. Western immunoblot analysis for determination of migration of recombinant CIA5 and CIA5 fragments during SDS-PAGE.

Supplemental Figure S6. Results from complementation experiments using intact and deleted *CIA5* genomic DNA constructs.

Supplemental Figure S7. Schematic diagram of mini-CIA5 construct.

Supplemental Figure S8. RT-PCR analysis of expression of genes with known responses to changes in CO_2 growth conditions.

Supplemental Table S3. Genes of interest and primer lists used for analysis of gene expression in *cia5* transformants complemented with either *CIA5* full length CDS (F-CIA5) or CIA5 partial CDS (mini-CIA5).

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