Augmented CO₂ tolerance by expressing a single H⁺-pump enables microalgal valorization of industrial flue gas

Hong Il Choi¹,4, Sung-Won Hwang¹,4, Jongrae Kim², Byeonghyeok Park³, EonSeon Jin², In-Geol Choi³ & Sang Jun Sim¹

Microalgae can accumulate various carbon-neutral products, but their real-world applications are hindered by their CO₂ susceptibility. Herein, the transcriptomic changes in a model microalga, Chlamydomonas reinhardtii, in a high-CO₂ milieu (20%) are evaluated. The primary toxicity mechanism consists of aberrantly low expression of plasma membrane H⁺-ATPases (PMAs) accompanied by intracellular acidification. Our results demonstrate that the expression of a universally expressible PMA in wild-type strains makes them capable of not only thriving in acidity levels that they usually cannot survive but also exhibiting 3.2-fold increased photoautotrophic production against high CO₂ via maintenance of a higher cytoplasmic pH. A proof-of-concept experiment involving cultivation with toxic flue gas (13 vol% CO₂, 20 ppm NOₓ, and 32 ppm SOₓ) shows that the production of CO₂-based bioproducts by the strain is doubled compared with that by the wild-type, implying that this strategy potentially enables the microalgal valorization of CO₂ in industrial exhaust.
apid increases in the atmospheric CO₂ concentrations have greatly impacted global ecosystems, and concomitant glo-
bal warming, climate change, and ocean acidification severely threaten all life on Earth. To address the issues caused by
increasing anthropogenic emissions, various carbon capture, utilization, and storage (CCUS) technologies have been developed
for CO₂ reduction. Microalgal-based CO₂ conversion, *inter alia*, is considered one of the most promising and self-sustainable
technologies because the relevant CO₂ sequestration process (i.e. photosynthesis) can be directly and entirely powered by solar
energy and the level of productivity is significantly greater than that obtained with other biological technologies. The wide range
of product portfolios is another merit of this platform. Despite their promising potential, the low tolerance of microalgae to high
concentrations of CO₂ is one of the crucial thresholds that pre-
cludes the real-world application of microalgae for CO₂ removal. In particular, this issue of CO₂ tolerance becomes more serious if
phototrophic microalgal cultivation occurs in concert with an industrial exhaust gas stream, which is the most economically and
energetically efficient biological process for removing CO₂ from air based on a life-cycle perspective because the proportion of
CO₂ in waste streams is commonly higher than 10%, a level that can significantly inhibit algal growth and unavoidably deteriorate
the biological CO₂ conversion efficiency. According to previous investigations, severe cytoplasmic acidification has been con-
sidered the principal reason for the impaired cell reproduction rate observed under high concentrations of acidic gas.

In recent decades, various efforts have been attempted to overcome the fatal weaknesses of microalgae. For instance, several
microalgal species have been newly isolated from native environ-
ments, and studies have indicated that these species may be able to thrive under very high CO₂ conditions. However, the
selection process is time-consuming and laborious, and these algae hold low economic potential compared with other compet-
titive strains due to insufficient information on the strain char-
acteristics and a lack of species-tailored gene modification tools, which could block further strain improvements. Adaptive
laboratory evolution approaches for improving the ability of algae to withstand high CO₂ environments through gradual acclima-
tion have been performed and represent efficient methods, but
the arduous and lengthy processes involved in this strategy are
also problematic. As an alternative, tolerance-enhancing trials
based on genetic engineering represent a game-changing tech-
nology with a high potential to confer unprecedented improved
tolerance, which can alleviate high CO₂ concentration-derived
acid stress. However, limited successful cases have been reported, and the tolerance enhancement effect has only been verified
under moderately high CO₂ conditions (5% CO₂), which is too
low to directly address industrial exhaust gases.

Therefore, CO₂-tolerant microalgal cell lines and relevant
strain improvement methods must be developed to expedite the realization and valorization of microalgae-based CO₂ reduction
technologies and products. Here, we show a genetic engineering-based strategy to improve the CO₂ tolerance of microalgae that could be broadly applied to various species. Based on the results of the transcriptional profiling of a model microalga, *Chlamydomonas reinhardtii*, under extremely high
CO₂ conditions, we identify the genetic principle of CO₂ tol-
erance. We demonstrate that the overexpression of a single
PMA gene resulted in greater than two-fold increases in the algal biomass and lipid productivities by directly converting
flue gas containing CO₂ and various acidic gases. We show that
this strategy improves the microalgal CO₂ fixation perfor-
mance, lowers the biomass production cost, and eventually
contributes to the establishment of profitable microalgae-based
CO₂ conversion technologies.

**Results**

**Transcriptome landscaping during high CO₂ conditions.** We first performed a RNA sequencing (RNA-seq)-based tran-
scriptome analysis of *C. reinhardtii* under extremely high CO₂
conditions (i.e. a 20% CO₂-enriched condition) to investigate its
cellular response to a high CO₂ environment and identify a gene
candidate that could augment CO₂ tolerance. In addition, transcriptome profiling of algae grown in a CO₂-independent
acidic environment (i.e. pH value of 5.0) was also carried out. The
stress-imposing conditions were determined according to the
results from preliminary condition screening experiments to determine the exposure levels of CO₂ and H⁺ that reduce the
growth rate by 50% (Supplementary Fig. 1). The RNA-seq analyses were then performed using six biological replicates of
each condition (Supplementary Fig. 2). Because H⁺ is known to
play a key role in CO₂ poisoning under high CO₂ conditions and
decrease the viability of algal cells (Fig. 1a), the latter analysis was
conducted as a benchmark to potentially elaborate the H⁺-related
CO₂ tolerance mechanisms even though they are unlikely to share
the exact same genetic response mechanism (Fig. 1b, Supple-
mentary Fig. 3, and Supplementary Data 1). The assay of the
specific growth rate under several high CO₂-mimicking condi-
tions (i.e. conditions with CO₂-independent low pH and a high
concentration of dissolved inorganic carbon (DIC); details are
provided in Supplementary Note 1) indeed suggested the exist-
ence of subtle differences in the cellular responses to acidic conditions depending on the presence and absence of CO₂. The
results showed that the decrease in the pH of the extracellular
environment caused by high CO₂ levels could be a minor toxic
factor, although it was not the only direct cause of the reduced
cell viability. This finding strongly implies that the interaction
between intracellularly absorbed CO₂ and cellular activity would
aggravate intracellular pH (pHₐ) acidification and thereby amplify
the toxicity (Table 1 and Supplementary Fig. 4). Namely, CO₂
intoxication seemed to be synergistically caused by an influ-
x of H⁺ from the acidic extracellular space as well as the intracellular
accumulation of dissolved inorganic carbon (DIC); details are
provided in Supplementary Note 1). In this context, the modulation of HSP expression is also an
important response during acclimation to high CO₂-derived acidic
conditions since the denaturation and aggregation of key proteins
(i.e. enzymes) by excessively generated H⁺ negatively contribute to
cell proliferation. Overall, 3867 and 1487 differentially expressed
genes (DEGs; log₂fold change (FC) > 1, p < 0.05) were identified
from the high CO₂ and low pH sets, respectively. Among these DEGs, 15.9% of the significantly upregulated and 18.1% of the downregulated genes in the high CO₂-treated cells were commonly found in the low pH-treated cells (Fig. 1c and Supplementary Data 1). These overlaps indicate that the algae may manage high CO₂-derived stress in a similar but not equal manner to counteract the stress attributed to low pH condition, and this conclusion is likely because the two inhibitory conditions may not exert identical toxic effects on the cell, as was experimentally revealed in this study (Table 1 and Supplementary Fig. 4).

Prior to the detailed inspection of individual gene expression levels, a Gene Ontology (GO) enrichment analysis was performed to extract and identify the biological processes (BPs) that are sensitively regulated in response to high CO₂ conditions. From
the 55 enriched GO terms, diverse modes of cellular responses for enduring the high CO2 condition were determined (Supplementary Data 2). Among the upregulated gene clusters under the high CO2 condition, GO-BP terms associated with the maintenance of cellular homeostasis (GO:0019725, GO:0006873, GO:0030003, and GO:0055082) were enriched, which may be attributed to heightened cellular efforts for minimizing the radical change in the intracellular condition caused by the extracellular condition. The enrichment of GO-BPs relevant to carbohydrate metabolism (GO:0044262, GO:0016051, and GO:0034637) was likely a response to the increased cellular activities against the high concentration of CO2 because carbohydrates are known to not only help adjust the intracellular osmotic pressure but also act as protective agents for maintaining cellular homeostasis. Additionally, under the high CO2 condition, protein and amino acid metabolism-related GO-BP terms (GO:0006520, GO:1901605, GO:0009072, GO:006551, GO:009100, GO:0006528, GO:0030163, GO:006511, GO:0019941, GO:0044257, and GO:0051603) were also found to be overrepresented, which was likely due to the activated cellular responses related to catabolizing proteins and amino acids that may lead to the production of NH3 and the reduction of intracellular H+ through deamination and decarboxylation, both of which are widely known as pivotal cellular mechanisms for microbial resistance to intracellular acidification.

Subsequently, the expression levels of the tolerance-related essential genes were assessed to verify the response behavior of the genes individually and to predict the detailed genetic cause of the low tolerance of C. reinhardtii to high concentrations of CO2 by comparing the purported cell responses with the actual gene expression patterns (Fig. 1d and Supplementary Data 3). As expected, a majority of essential genes that constitute the CCM were significantly downregulated (16 out of 21 investigated genes) under extremely high CO2 conditions as a counteraction to the increased environmental DIC concentration. Because an increased extracellular DIC concentration can trigger a series of reactions that ultimately results in excessive endomembrane acidification by the accelerated inward flux of DIC species and their intracellular hydration, which promotes additional generation and accumulation of H+ in the cytoplasm, this gene regulation seems to be an example of a cellular endeavor against high CO2 conditions (Supplementary Fig. 5). Countertrends were found for CAH2, RHP1, and RHP2, and the plausibility of this hypothesis has been previously validated. Interestingly, although the expression of CCM genes has been known to be mainly influenced by the external CO2 concentration, our findings showed that many CCM components (CAH1, CAH4, CAH5, HLA3, LCIA, CCP1, CCP2, LCID, and LCIE) expressed in response to low pH conditions showed similar patterns (i.e. downregulated) to those found under the high CO2 condition, which likely indicates that several CCM genes are also related to the response to CO2-independent low pH conditions. Regarding the determinants of cell components, the expression of key enzymes involved in controlling the composition of the cellular membrane appears to be regulated under extremely high CO2 conditions. For example, upregulated expression was observed for SQD3, which contributes to a decrease in the membrane fluidity of the cell envelope by increasing the composition ratio of saturated membrane lipids. In addition, the results showed increases in the expression levels of Cre08.g365950, Cre08.377300, Cre12.g521650, and DGT1, which are responsible for converting unsaturated lipids in intracellular and plasma membrane (PM) regions into triacylglycerol (TAG), a typical energy storage sink synthesized under various stress conditions. Consequently, these responses may inhibit the entry of H+ into the cell, which decreases passive proton influx and eventually leads to stress evasion.

Additionally, as expected, under high CO2 conditions, various HSPs from a wide range of HSP subfamilies were highly expressed. These increases in the expression levels of stress-inducible molecular chaperones may contribute to the turnover and refolding of proteins that are denatured and aggregated by excessive H+–derived oxidative stress. Meanwhile, a higher expression of a number of building blocks for ATP synthase was also observed under harsh conditions. In particular, mitochondrial ATP synthase-associated (ASA) proteins, such as ASA1, ASA3, and ASA4, which are involved in the dimerization of ATP synthase, showed 2.93-, 1.59-, and 1.63-fold upregulation (all p values were less than 0.01), which in turn can result in increased ATP synthesis activity of the ATP synthase complex. These changes may correspond to cellular activity to increase ATP synthesis because acidic conditions lead to the consumption of ATP for the...

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## Table 1 Comparison of the specific growth rate and concomitant pH of *C. reinhardtii* culture under high CO2-related stress application conditions.

| Condition       | μ (d⁻¹) | pH     | Low pH | High [DIC] |
|-----------------|---------|--------|--------|------------|
| Ambient (control) | 1.253 ± 0.007 | 6.92 ± 0.04 | 1.162 ± 0.105 | 1.387 ± 0.005 |
| High CO2        | 0.664 ± 0.004 | 6.23 ± 0.01 | 6.25 ± 0.05 | 7.32 ± 0.01 |
| Low pH          | 1.162 ± 0.105 | 6.25 ± 0.05 | 7.32 ± 0.01 | 7.32 ± 0.01 |

The data are shown as the average values ± standard deviations of the mean (n = 2). Data are mean ± SD of two biological replicates. DIC means dissolved inorganic carbon. The conditions for low pH (pH 6.2) and high [DIC] (7 mM) were determined by the corresponding [H+] and [DIC] values under high CO2 (20%) conditions, respectively. Source data are provided as a Source Data file.
operation of H\textsuperscript{+}-pumps and the activation of ATP-dependent HSPs, among other processes\textsuperscript{2,25}.

Under extremely high CO\textsubscript{2}, the CO\textsubscript{2}-intolerant species \textit{C. reinhardtii} struggles to maintain its cytosolic neutrality by dynamically modulating genes associated with CCM, cell composition modification, protein recovery, and ATP synthesis in addition to previously elucidated CO\textsubscript{2} tolerance mechanisms. In contrast, the gene expression of H\textsuperscript{+}-pumps (e.g. PMAs, vacuolar H\textsuperscript{+}-ATPase – V-ATPase, and H\textsuperscript{+}-pyrophosphatase)\textsuperscript{76} profiled under high CO\textsubscript{2} conditions reveals that H\textsuperscript{+} sequestration from the cytoplasm to the extracellular region or contractile vacuole does not seem to be adequately regulated (i.e. not significantly upregulated) in the microalgae, which is clearly different from previously reported expression trends observed with acid- and CO\textsubscript{2}-tolerant microalgal species\textsuperscript{7,11,13}.

Rather intriguingly, a decrease in the expression of the PMAs – PMA2 (Cre10.g459200; slightly downregulated) and PMA3 (Cre03.g164600; highly downregulated with statistical significance) – was observed under high CO\textsubscript{2} conditions, which is an important finding because PMAs play a crucial role in life support during exposure to various acidic conditions\textsuperscript{13,19}.

The reproducibility of the gene expression results at the transcriptomic and translational levels under high CO\textsubscript{2} conditions was verified by qRT-PCR and immunoblotting (Supplementary Figs. 6a–c and Supplementary Table 1), respectively, which implies the mRNA-protein expression correspondence and, at the same time, suggests that the downregulation can directly influence the congenital low tolerance. Although their distinctive functions have not yet been explicated, PMA2 and PMA3 are two PMA proteins identified in \textit{C. reinhardtii} with a sequence similarity of 47.4\%, and both of these proteins have highly conserved regions that are generally found in well-characterized PMAs (Supplementary Fig. 7)\textsuperscript{27–29}.

Since PMA is a key and primary molecular workhorse located at the forefront of the cell and responsible for adjusting the intracellular H\textsuperscript{+} concentration by pumping out H\textsuperscript{+} into the extracellular region, which results in controlling the pH\textsubscript{1} and generating an electrochemical gradient to offer an H\textsuperscript{+}-mediated driving force for various secondary transport proteins\textsuperscript{19,26}, the atypical expression of PMAs under high CO\textsubscript{2} conditions may limit the discharge of excessive H\textsuperscript{+} out of the cell, which would lead to undesired H\textsuperscript{+} accumulation, even more acidification of the intracellular region, and disruption of the proton gradient for nutrient and ion transport. All of these changes can inhibit normal cell propagation (Fig. 1a and Table 1).

Therefore, we conjectured that the CO\textsubscript{2} intolerance of the alga can be attributed to insufficient expression of PMA genes and the concomitant low ability of the cell to extrude the excessive H\textsuperscript{+} generated under external CO\textsubscript{2} conditions. We thus hypothesized that the overexpression of PMAs would improve the CO\textsubscript{2} tolerance of the alga by increasing proton efflux and improving the ability of the alga to maintain its endomembrane pH at near neutrality even under high CO\textsubscript{2} environments.

**Construction of microalgal strains with high CO\textsubscript{2} tolerance.** To augment the CO\textsubscript{2} tolerance of \textit{C. reinhardtii} by compensating for the low expression of H\textsuperscript{+}-ATPase, the overexpression target was a typical PMA, i.e. plasma membrane H\textsuperscript{+}-ATPase isoform 4 (hereafter referred to as PMA4) from \textit{Nicotiana plumbaginifolia}, and its features have been well characterized and successfully expressed in various bioplatforms, such as other plants and yeasts\textsuperscript{30,31}.

If this pump can be activated in algal systems, the exogenous gene expression strategy can provide several merits. Because of its host-independent property, the expression of PMA4 holds the potential to be a versatile approach for various microalgal species suffering from stagnant H\textsuperscript{+} extrusion and consequent CO\textsubscript{2} intolerance, which is a general but critical issue that interferes with the practical application of microalgae-based CO\textsubscript{2} conversion technology\textsuperscript{3,8}.

In addition, PMA4 could help prevent cosuppression, which often occurs in homologous overexpression cases\textsuperscript{32}.

Given these findings, the gene insert was designed for mutagenesis. Because deletion of the autoinhibitory domain (located in the C-terminus; Supplementary Fig. 7), which is regulated by interacting with the regulatory 14-3-3 proteins (located in the microalgae, \textit{C. reinhardtii}), increases the activity of the H\textsuperscript{+}-pump\textsuperscript{33,34}, we decided to use the truncated form of PMA4 (referred to as PMA4ΔCter) instead of the full-length protein to confer the greatest acid-derived tolerance to the microalgal cell.

Subsequently, to label a reporter for probing the subcellular localization of the expressed acid-secreting nanomachine, the PMA4ΔCter coding gene was translationally fused at its 3'-coding-end to the monomeric Venus fluorescent protein (mVenus) coding gene with a flexible linker sequence (GGSGGGSG) (referred to as PMA4ΔCter-V) and placed under the control of a strong constitutive promoter (Hsp70A-RbcS2) (Fig. 2a and Supplementary Fig. 8a). Afterwards, the codon-optimized insert cassette was delivered into the cell by electroporation.

After transformation, two independent mutants were screened — whose genetic and phenotypic stabilities are maintained over time — from selective medium plates containing Zeocin via colony PCR (Fig. 2b) using a designed screening primer set (Supplementary Table 1 and Supplementary Method 1). The transgenic cells were named PMA4ΔCter-V4 and PMA4ΔCter-V10 according to the order of their isolation. Due to the lack of an efficient homologous recombination method for \textit{C. reinhardtii}\textsuperscript{34}, the inserted gene was randomly intercalated into the genome. Gene insertion leads to disruption of the original genome sequence, which can impact transgenic cell phenotypes. To clarify the cause of the phenotypic changes (vide infra), insertion site analysis was performed on the isolated strains by next-generation sequencing (NGS)-based whole-genome sequencing using HISAT2 software (Fig. 2c; see Supplementary Method 2 for details). In the PMA4ΔCter-V4 strain, only one insertion site was identified in chromosome 7, and this mutation deleted 23 base pairs (bps) from the genome and disrupted a predicted protein-encoding gene (Cre07.g331114). In PMA4ΔCter-V10, one inserted gene was also found on chromosome 6. During the insertion process, an approximately 5.5-kbp truncation of the genome occurred, and three protein-encoding genes, tail-specific protease 2 (TSP2; Cre06.g265850), a predicted protein (Cre06.g265900), and dynein heavy chain 3 (DHC3; Cre06.g265950), were unintentionally lost.

The insertion site revealed by NGS was visually confirmed by PCR using appropriate primers (Supplementary Fig. 8b, c and Supplementary Table 1). Given the annotations of the disrupted genes (Supplementary Table 2) and previous reports on their functions\textsuperscript{35–37}, it is unlikely that the gene deletion events directly affected the ability of the transgenic strains to maintain their pH\textsubscript{1} against acid-derived CO\textsubscript{2} stress, which implies that the phenotypic modifications described below are generally not associated with the lost genes but rather caused by exogenous PMA expression. Following this analysis, the expression of the inserted gene was confirmed by quantitative real-time RT-PCR (Fig. 2d) using a designed primer set (Supplementary Table 1 and Supplementary Method 3), and the successful transcription of mRNA encoding PMA4ΔCter-V in the transgenic strains was confirmed by cDNA amplification. Moreover, no expression of PMA4ΔCter-V was detected in the wild-type (WT) strain.

The expression of the protein of interest was then verified by Western blotting, and a band at approximately 130 kDa (Fig. 2e), which is
located near the expected molecular weight of the PMA4ΔCter-V protein (120.81 kDa; estimated from its sequence), was visualized only with the transgenic strains when the same amount of protein is loaded (i.e. 20 μg), which reveals PMA overexpression properly occurred in the mutants (see Supplementary Method 4 for details). In addition to molecular-level expression tests, whether the protein is expressed in the intended subcellular part (i.e. PM) should be determined before evaluating the cellular performance because the protein must be localized appropriately before it can pump H^+ into extracellular space. The localization of the protein was examined with the aid of a fluorescent tag protein (i.e. mVenus) (see Supplementary Method 5 for details). For precise fluorescence comparison, a transgenic microalgal cell line expressing only mVenus (referred to as V8) was constructed using a separate insert cassette (Supplementary Fig. 8d) as a fluorescence reference strain. The integration of the transgene was genotypically verified (Supplementary Fig. 8e). First, the quantitative fluorescence of the cell lines was estimated by flow cytometry using BD AccuriTM C6 Plus software and Flowjo software (Fig. 2f). The results showed that mVenus expression induced significant shifts in the fluorescence peaks toward a higher intensity in the transgenic algal strains, including PMA4ΔCter-V4, PMA4ΔCter-V10, and V8 (Supplementary Table 3 and Supplementary Fig. 9e). Subsequently, the localization of the fluorescent source in C. reinhardtii was specified by confocal microscopy. The image-based assessment visually demonstrated that bright fluorescent proteins were preferentially concentrated near the PM of PMA4ΔCter-V4 and PMA4ΔCter-V10, whereas no notable concentration of fluorescence in the specific subcellular region was detected in the WT (Fig. 2g and
Based on the previous pH tolerance evaluation, an autotrophic CO₂ tolerance test (without the organic carbon source) was performed with the cell lines after exposure to an extremely high CO₂ concentration (20%) to exclude the effect of other influencing factors on acid tolerance. Although the CO₂ concentration was somewhat higher than that found in typical industrial exhaust gas⁶, it was selected to test whether the strains possessed sufficient acid tolerance to be used in practice given the additional acidification effect that may be induced by the various acidic gaseous components (e.g. SOₓ and NOₓ) contained in general industrial exhaust gas streams. In this assay, the algal biomass production rates estimated from the turbidities of the cultures were compared to accurately assess CO₂ sequestration-related performance metrics because the microalgal CO₂ fixation rate (R_CO₂) is directly proportional to biomass productivity². For the estimation, an OD₆₀₀-biomass correlation curve (Supplementary Fig. 12) and Eqs. (2) and (3) were used. According to the tolerances test, the WT strain showed stagnant biomass productivity (especially in the initial culture phase; Supplementary Fig. 11a) when exposed to high CO₂ concentrations compared with its growth under ambient conditions, which clearly reveals the vulnerability of these algal cells to elevated CO₂ levels. According to the entire batch culture result, eventually, the WT showed 0.66-fold decreased maximum biomass production while exhibiting 1.09-fold increased productivity under high CO₂ conditions (by Day 6) compared to those under ambient CO₂ conditions (by Day 17) during reaching their maximum cell growths (Fig. 3c and Supplementary Fig. 11c). The higher productivity is mainly attributed to the relatively shorter cultivation period because of the rapid cell death by high-CO₂ stresses. However, the slight increase would be hardly meaningful in terms of the CO₂ removal because the autotrophic growth rate that is not correspondingly increased against the elevated supply of CO₂ to the algal culture, inevitably causes the generation of unixed CO₂, which significantly lowers the overall CO₂ reduction efficiency. Moreover, the dramatically reduced maximum cell density would also be unfavourable from the same point of view, because the final biomass concentration is one of the most influential factors affecting the economic feasibility and energetic efficiency — which is intimately related to the net CO₂ removal ability of CO₂ conversion processes — from the life-cycle perspective²⁴.⁴⁰.

On the contrary, the transgenic microalgal strains exhibited noticeable improvements in any photosynthetic production metrics, including maximum biomass productions and rates, even in a culture system to which an extremely high level of CO₂ was provided. Their maximum biomass productions were considerably above that found for the WT strains under identical CO₂ conditions, and 3.22- and 3.03-fold improvements were provided for PMA4ACter-V4 and PMA4ACter-V10, respectively (upper panel of Fig. 3c). The accumulative productions and the rates of the transgenic strains under high CO₂ conditions (by Day 7) were also much higher (rather 1.12- and 1.09-fold improvements in the accumulative biomass productions and 2.77- and 2.62-fold improvements in the productivity for PMA4ACter-V4 and PMA4ACter-V10, respectively) than those under ambient CO₂ conditions (i.e. autotrophic pH 7.0) (by Day 17) (Fig. 3c and Supplementary Fig. 11c), which illustrates that the PMA-expressing mutants can even benefit from the same point of view, because the final biomass concentration is one of the most influential factors affecting the economic feasibility and energetic efficiency — which is intimately related to the net CO₂ removal ability of CO₂ conversion processes — from the life-cycle perspective²⁴.⁴⁰.

Characterization of the performance of the transgenic cells. Since the CO₂ tolerance improvement strategy described in this study is in line with the approach of enhancing the tolerance to low pH conditions, the productivity of the low pH-grown cell lines was evaluated first. The evaluation of the pH tolerance of the algal cells was conducted under two independent cultivation modes: mixotrophic and autotrophic conditions. For the mixotrophic culture of the facultative autotroph, acetic acid was applied as an organic carbon source. At a pH value of 5.5, the transformed cell lines showed a clear difference in growth performance compared with the WT cells. In particular, under mixotrophic cultivation at pH 5.5 (Fig. 3a, b), the proliferation rate of the WT cells was significantly stunted, and the productivity of the transgenic strains was high, which indicated that the acid tolerance of the mutants was successfully augmented. Because the growth of the transgenic strains at this low pH was comparable to that at neutral pH (Supplementary Figs. 10a and 11a), this constitutive PMA overexpression strategy did not appear to have a negative effect on the cells. In the photovoltaic acidic cultivation, the exogenous PMA-expressing mutants also exhibited a relatively improved growth performance under the same acidity (pH 5.5) but did not show a dramatic growth difference compared with the results obtained with the corresponding mixotrophic culture (Supplementary Figs. 10b, c and 11b, c). This trophism-dependent growth performance difference can be ascribed to the fact that the membrane-permeable weak acid protonated acetate (i.e. acetic acid), which was added to create the mixotrophic conditions, may exacerbate intracellular acidification by generating additional H⁺ in the microalgal cells.⁸ In this context, the acetic acid-supplemented pH 5.5 environment can inflict markedly higher levels of acid stress on the cells than a medium with an identical pH but without acetic acid, and this higher stress will eventually lead to the apparently dramatic difference in tolerance observed between the two different carbon regimes (Fig. 3b and Supplementary Fig. 11a–c). These results thus imply that the engineered cells possess high tolerance not only to extracellular acidic conditions but also to factors that aggravate pH acidification.
Fig. 3 Cell performance of the transgenic strains compared with the WT strain. **a** Spot culture with serial dilution on agar medium (supplemented with acetic acid) at pH 5.5. **b** The pH tolerance during liquid cultivation (pH 5.5; supplemented with acetic acid) is markedly enhanced via PMA4ΔCter expression. Shown are data from duplicate cell culture. The cell states at Day 2 of WT, PMA4ΔCter-V4, and PMA4ΔCter-V10 are displayed from the far left of the top panel. **c** The photoautotrophic biomass production (upper panel) and CO2 fixation rate (RCO2; lower panel) are improved under extremely high CO2 conditions (20% CO2). The two graphs share the same timescale. The arrows indicate the time points (distinguished by the colors) that the cell lines reached their maximum concentrations. Data from duplicate cell cultures are shown. Each dot in the lower panel depicts two biological replicates. **d** Per-cell gross photosynthetic rate of the strains exposed to high CO2. The parameter was estimated as light-dependent O2 evolution plus dark respiration. The double asterisks indicate statistically significant differences in the gross photosynthetic rate of each strain under the high CO2 environment compared with those under the ambient conditions (p < 0.05). Each bar represents the mean ± SD of three independent experiments. **e** In vitro ATPase activity assay using isolated PM-enriched fraction samples. The double (p < 0.05) and triple (p < 0.01) asterisks denote statistically significant differences compared with the ATPase activity of the WT strain, respectively. Each bar represents the mean ± SD of four independent experiments. In **d**, **e**, and **f**, the statistically significant differences were determined by a two-tailed Student’s t test. **g** Representative image of bromocresol purple-mediated in vivo ATPase activity test. Source data are provided as a Source Data file.
values compared with the WT strain, respectively, during their exponential growth phases when their effective CO₂ fixations occur (between Day 2 and 6). Taken together, this tolerance improvement strategy raises the mutants’ maximum biomass concentrations during a shorter culture period under high CO₂ supply conditions and improves their CO₂ removal rates and efficiencies, all of which are requisites of microalgal strains for efficient CO₂ sequestration and economically feasible CO₂-derived product productions.

In parallel to the growth assessment, the per-cell basis photosynthetic O₂ evolution of the cell lines exposed to high CO₂ conditions was evaluated (see Supplementary Method 6 for details). Consistent with the productivity data, the high CO₂-grown WT strain exhibited a lower gross photosynthetic rate (i.e. O₂ evolution plus dark respiration) than the WT strain cultivated under ambient conditions (approximately 50.4% decrease), which implies that the algal photosynthetic apparatus was severely impaired by the high CO₂ condition. In contrast, the PMA4ΔCter-V4 and PMA4ΔCter-V10 strains grown under high CO₂ conditions showed an increased photosynthetic O₂ evolution rates in comparison to those found during growth under atmospheric conditions (approximately 58.6% and 37.3%, respectively) (Fig. 3d). Based on observations from previous reports and the results of this study, this excess CO₂-related photosynthesis-related enzymes under tolerably high CO₂ conditions to facilitate biological CO₂ assimilation, as has been well documented. Thus, C. reinhardtii, which is a congenitally CO₂-intolerant microalga, became resistant to high CO₂ conditions via PMA expression and its activated core machinery for photosynthesis was thereby able to generate an increased amount of oxygen.

To probe whether PMA expression indeed improves photosynthetic O₂ evolution and biomass productivity by providing cells with the ability to maintain their pH under neutrality, we next monitored the time-lapse cytoplasmic pH changes after high CO₂ treatment using a membrane-permeable pH-sensitive fluorescent dye, BCECF, AM (2',7'-bis(2-carboxyethyl)-5 (6)-carboxyfluorescein, acetoxymethyl ester) (see Supplementary Method 7 for details). To eliminate the background signal, including the autofluorescence of the cells (for all strains) and the fluorescence of the fluorescent probe (i.e. mVenus for the transgenic algal lines), from the fluorescence of interest emitted by the pH-sensitive dye, the pH of each algal strain was calibrated independently (Supplementary Fig. 13). Consequently, the WT strain showed a dramatic decline in their pH during 2 h of exposure to high CO₂ (a median pH value of 6.56), whereas the transgenic cell lines showed a better ability to robustly maintain a near-neutral pH under acidic conditions (median pH values of 6.65 and 6.78 were obtained for PMA4ΔCter-V4 and PMA4ΔCter-V10, respectively) (Fig. 3e), which demonstrates that high CO₂ conditions largely affect the viability of microalgae by lowering the cytosolic pH and that PMA expression in algae results in a positive response to CO₂-derived intracellular acidification. Given that the photosynthetic CO₂ fixation system and O₂ evolution machinery are highly sensitive to the pH and CO₂ conditions of the surroundings as well as to the cytosolic and subcellular pH, the differences in the ability of the cells to maintain their pH under high CO₂ conditions appear to be the fundamental cause of the tolerance gap between the WT and transgenic strains.

In the final stage of the cell performance characterization, we investigated the H⁺ pumping ability of the algal cells determined by the activity of ATPase on the PM region to determine the CO₂ tolerance conferred to the mutants by exogenously expressed PMA via the H⁺ extrusion function of the cell. This ability was comprehensively evaluated both in vitro and in vivo. In the in vitro ATPase activity test, the PM fraction-enriched protein sample, which is identical to that isolated for assessment of its localization (i.e. ELISA), was used (see Supplementary Method 8 for details). As expected from the results of the previous performance tests, PMA4ΔCter-V4 and PMA4ΔCter-V10 exhibited approximately 2.5- and 2.6-fold higher ATPase-mediated ATP hydrolysis performance than the WT strain, respectively, which demonstrates that the transgenic strains have substantially higher H⁺ translocation activity in their PM regions than the WT strain (Fig. 3f). The greater ATPase activity in the mutants could cause higher ATP consumption, which may lead to additional energetic costs associated with the exogenous ATPase. However, given algal growth under neutral conditions (Fig. 3b and Supplementary Fig. 11a, c), the viability of the algal cells was not significantly affected. An in vivo assay was performed to confirm the algal H⁺ extrusion performance at the cell level and the direction of the H⁺ flux using a chemical pH indicator, bromocresol purple (see Supplementary Method 9 for details). The colourimetric assay visually demonstrated that the H⁺ flux was appreciably improved in the PMA-expressing mutants, as can be inferred from the color change on the perimeter of the cells caused by the expelled H⁺ and the accompanying extracellular acidification (Fig. 3g and Supplementary Fig. 14), and this result also provides direct evidence of the outward flux direction of H⁺, namely, from the intracellular region to the exofacial space, and hints that the H⁺ pumping protein was properly localized and oriented as desired. On balance, the data clearly show that the expression of the H⁺-pump imbued the algal transformants with markedly improved H⁺ efflux and an augmented ability to maintain their pH against factors causing severe intracellular acidification (e.g. membrane-permeable organic acids and CO₂); thus, the algae showed enhanced tolerance to extremely high CO₂ conditions.

Directly utilizing coal-fired flue gas for fuel production. To estimate the real-world practicality of the engineered algal strain as a CO₂-derived biofuel production platform, we grew the PMA4ΔCter-V4 strain, which showed remarkable productivity under the high CO₂ level (Fig. 3c), in the presence of actual coal-fired flue gas discharged from the Taeon thermal power station complex unit 5 boiler of Korea Western Power Co., Ltd. The gas stream consisted of approximately 13% CO₂, 20 ppm NOₓ, and 32 ppm SOₓ on average (vol.%) in this outdoor cultivation, coal-fired flue gas was chosen as the exemplary industrial flue gas because the discharge of CO₂ from the exhaust is the largest contributor to global emissions, and its reduction is therefore vital. Despite the urgency of mitigating this pollutant, the direct application of microalgae has been considered impractical to date due to its vulnerability to CO₂. The strain was phototrophically cultivated with continuously provided CO₂ through the introduction of highly acidic exhaust gas as the sole carbon source in a microagal CO₂ bioconversion facility constructed next to the coal combustion boiler using a 10 L (in working volume) photobioreactor (PBR) (Fig. 4a). After two weeks of cell culture, we holistically evaluated various performance indices of the strain using Eqs. (2)-(6). We observed that PMA4ΔCter-V4 exhibited a markedly improved phototrophic growth rate than the WT strain (2.28-fold higher) and beneficially converted the high concentration of CO₂ included in the noxious gas stream. The increased biomass productivity in the transgenic algal strain led to the enhancement...
Fig. 4 Proof-of-concept biomass and biofuel production with the engineered strain PMA4/Cter-V4 using coal-fired flue gas. a Bioconversion of CO₂ in the flue gas stream emitted from the coal-fired power plant. A house-manufactured bubble column PBR (10 L of working volume) was employed for cultivation in the vicinity of the coal combustion boiler. b Radar plot showing various cellular performances measured in duplicate (n = 2). The covered ranges on the axis indicate the standard deviation of each parameter. Source data are provided as a Source Data file.

Discussion

To date, numerous studies have attempted to address the low CO₂ tolerance issue, which is a key hurdle that reduces the applicability of microalgal CO₂ reduction technology. Due to the lack of comprehensive analyses of the mechanisms underlying the intolerance of microalgae, previous approaches only focused on resolving the incidental causes of vulnerability; thus, the resulting improvements did not meet the requirements for practical applications with various types of industrial flue gas. Therefore, we thoroughly inspected the global gene expression patterns of microalgae in response to extremely high CO₂ conditions using the model microalga C. reinhardtii to specify the immediate reason for its susceptibility to acidic gas. The investigation revealed that the representative intolerant species exhibited a relatively low expression level of the core H⁺ pumping system, which is inconsistent with the previously reported response mechanisms to high CO₂-derived stress observed in CO₂-tolerant microalgae. Notably, PMAs, which regulate the pH, were rather downregulated (PMA2 was slightly downregulated and PMA3 was significantly downregulated); thus, we postulated that aberrant expression of the H⁺-pumps is the main factor for the ongoing intracellular acidification, which ultimately leads to a severe decline in cell viability. The reason for the unpredicted gene expression levels may not be clearly explained only by the investigations performed in this study, but one speculation — regardless of the exterior acidity (i.e. both high CO₂ and CO₂-independent low pH conditions impose acidic stress on the cell), the expression of the PMA genes in the CO₂-intolerant alga is always negatively regulated by the abundance of extracellular CO₂ as if the genes are constituents of CCM – could provide evidence of the condition-dependent gene expression patterns detected in this study (Supplementary Fig. 6a, b). A possible dependence of PMA expressions on the extracellular CO₂ concentrations was also suggested by a recent comprehensive proteomic study of C. reinhardtii. The results showed that PMAs (both PMA2 and PMA3) of the alga may be subordinate to the CCM and intimately interact with its core elements (e.g. HLA3 and LCI1). Since the gene regulation of CCM and its components is heavily influenced by the external CO₂ conditions, the functional relevance exhibits the possibility of an effect of exterior CO₂ on PMA expression. However, additional studies are needed to precisely conclude the association of the two machineries (i.e. the H⁺-pump and carbon concentrating system). In parallel, characterization of the distinctive biomolecular properties of algal PMAs, such as their putative 14-3-3 protein-independent auto-inhibitory mechanisms, merits further study to provide a better understanding of the proteins and furthermore the organism, which would ultimately facilitate the greatest use of the microorganism’s potential abilities (Supplementary Fig. 7).

As predicted from the transcriptomic analysis, we introduced a typical PM-bound H⁺ transporter, whose expression is initiated by a strong constitutive promoter to enable continuous PMA expression with the low possibility of intervention by the host’s gene expression controlling mechanisms (e.g. CCM, vide supra), into congenital CO₂-susceptible microalgae to ameliorate the intolerance problem and rigorously verified the characteristics of the generated transgenic microalgae. The WT strain showed a reduced autotrophic growth rate under high CO₂ conditions compared with that under ambient conditions, which may be attributed to its high CO₂-labile pH stability and the resulting damage to its photosynthetic activity. In contrast, the CO₂ tolerance of both mutant strains was clearly augmented, which resulted in markedly increased biomass productivity even under harsh conditions by facilitating H⁺ efflux, thereby reinforcing the pH maintenance ability and contributing to the activation of oxygenic photosynthetic apparatuses in combination with sufficient DIC from the surroundings. In the proof-of-concept experiment, microalgal biofuel production using a coal-fired flue gas
gas with a high level of CO$_2$ was investigated, and the engineered algal strain exhibited over twofold increases in CO$_2$-originated biomass and biofuel productivities without any process intervention, which may negate the reduction in CO$_2$ efficiency of the overall system. These findings demonstrate the applicability of microalgae as a practical platform for the conversion of CO$_2$ in the flue gas to produce diverse sustainable products. Strain improvement technology can mediate the utilization of highly cytotoxic waste streams as a freely serviceable carbon source for microalgal cultivation and will thus aid the use of microalgae as a biological platform for practical CO$_2$ reduction.

In addition to the environmental benefit, the enhanced tolerance and resulting improvements in biomass and lipid productivity can contribute to promoting the economic feasibility of microalgal systems, including the realization of the price of biofuel. According to previous studies, 2.28- and 4.68-fold increases in biomass and biodiesel-convertible lipid (i.e. FAME) productivity can be anticipated in biomass and biodiesel-convertible lipid (i.e. FAME) productivity are anticipated to decline by exponential factors of 1.07 and 0.88, respectively. The upgraded productivities will undoubtedly serve as a catalyst for lowering the production cost of microalgal biofuel in various forms, expanding the market base of biofuel, and ultimately valorizing the overall microalgal industries. The need to use closed cultivation systems (i.e. PBRs) for biocontainment to prevent undesired dissemination of genetically modified (GM) microalgae to the environment and the resulting increase in production costs can be a concern. Even though the cultivation of GM algae in open environment does not seem to be accompanied by greater environmental risks than expected, public concerns are not negligible. Therefore, PBR systems should be inevitably exploited for the cultivation of GM algae. In fact, according to an extensive techno-economic analysis study of microalgal cultivation, PBRs can be used as equipment for moderating the price of products in the near future with ongoing technical developments. Their use obviously ensures better photosynthetic efficiency and shorter light paths, which are key parameters lowering the production costs of biomass. These properties eventually contribute to improving the economic feasibility of the process by counterweighting the costs related to the installation and operation of PBR. In addition, considering the goal of the cultivation — practical CO$_2$ reduction with phototrophic microalgal culture — PBRs constitute the most suitable platform for serving the purpose of enabling intensive bioconversion of CO$_2$. Thus, harnessing well-designed and cost-effective PBR systems may simultaneously guarantee thorough containment of GM organisms, efficient CO$_2$ fixation, and high biomass productivity while securing economic competitiveness.

We found that the expression of exogenous PMA is beneficial for algae because it significantly improves their tolerance to acidic conditions (i.e. low pH and high CO$_2$) and does not compensate for growth under neutral pH conditions. The simple structure of PMAs — i.e. the protein comprises a single polypeptide — seems to play a pivotal role in successful PMA expression and concomitant tolerance enhancement without burdening the transgenic cells. By virtue of this structural property, it is likely that the desired augmentation was directly achieved by overexpression of a single protein unit. In contrast, straightforward tolerance enhancement would have been quite challenging if the overexpression target was a gene encoding a subunit of a multisubunit H$^+$-pumping protein such as V-ATPases. Since such proteins are congenitally composed of several subunits and normally function only when the complex is well organized with the proper quality and quantity of subunits, a possible approach that involves the overexpression of several specific subunits would lead to protein malfunction due to abnormal subunit assembly and ultimately thwart the improvement attempt. A strategy that aims at overexpressing an entire protein complex (i.e. individual overexpression of an entire subunit) could also be problematic because multigene overexpression may impose an enormous genetic burden on the transgenic cell line. Despite the expected difficulty and intricacy, uncovering the V-ATPase function in CO$_2$ tolerance remains an interesting topic for future work because V-ATPases are also known to play an important role in the maintenance of the pH$_i$ against extracellular acidic conditions.

Another advantage of this strategy is that the single H$^+$-pump can be universally expressible in various organisms and causes a dramatic increase in the algal CO$_2$ tolerance. Thus, this simple but highly effective improvement strategy is likely applicable to multiple algae and not limited to a particular species. In particular, the ability to enhance robustness will be greatly effective for programmable microalgal chassis because potential cell factories can be easily transformed to phototrophically produce various bioderived sustainable products by additional transformation but are generally unable to tolerate environmental stresses at practical levels. On the one hand, this acid tolerance augmentation strategy could also be conjugated as a genetic approach for the maintenance of axenic conditions in acidic microalgal cultivation due to the considerably improved resistance toward an extracellular acidic pH. Taken together, the results strongly provide a plausible and versatile solution for overcoming vulnerability to CO$_2$, a fundamental hurdle of microalgae-based CO$_2$ conversion technology, and thereby expedite the realization of CO$_2$ reductions systems and the commercialization of microalgal-derived products.

**Methods**

**Microalgal strain, media formulation, and culture conditions.** *C. reinhardtii* WT strain CC-125 was obtained from the Chlamydomonas Resource Center at the University of Minnesota. All the engineered cell lines used in this study were constructed from this background strain. Laboratory-scale culture of the algae was conducted with modified Tris-acetate-phosphate (TAP; hereafter referred to as MTAP) and Tris-phosphate (TP; hereafter referred to as MTP) media, and the buffer capacities of the media were enhanced with an additional buffer material, namely, MES, to strictly maintain the acidity of the culture. Otherwise, the pH would be seriously lowered due to ammonia uptake, which can prevent accurate evaluations of CO$_2$ tolerance (Supplementary Fig. 4). The mixotrophic MTAP medium at neutral pH consisted of 10 mM MES, 17.4 mM acetic acid, 7 mM NH$_4$Cl, 0.83 mM MgSO$_4$·7H$_2$O, 0.45 mM CaCl$_2$·2H$_2$O, 1.65 mM K$_2$HPO$_4$, 1.05 mM KH$_2$PO$_4$, 0.134 mM Na$_3$EDTA·2H$_2$O, 0.136 mM ZnSO$_4$·7H$_2$O, 0.184 mM H$_2$BO$_3$, 40 mM MnCl$_2$·4H$_2$O, 32.9 mM FeSO$_4$·7H$_2$O, 12.3 μM CoCl$_2$·6H$_2$O, 10 μM CuSO$_4$·5H$_2$O, and 4.44 μM (NH$_4$)$_2$MoO$_4$. The pH was adjusted to a value of 7.0 with 25 mM Tris base. All chemicals were purchased from Sigma-Aldrich (USA).

Moreover, an autotrophic MTP medium (pH 7.0) was prepared using a method identical to that used for the MTAP formulation, although 0.35% HCl was added instead of acetic acid. The cells were grown at 23°C in shaking incubators at a rotation rate of 120 rpm under continuous light illumination (30 μE m$^{-2}$ s$^{-1}$). The headspaces were provided with air and 20% CO$_2$-enriched air according to the purpose of each experiment. Cell growth was monitored daily using a UV-1800 UV spectrophotometer at 800 nm (Shimadzu, Japan). To search for the factor underlying high CO$_2$ toxicity, media with CO$_2$-independent low pH (pH 6.2) and high [DIC] (7 mM) were prepared based on MTP medium. The pH was modified by the addition of HCl and the [DIC] was adjusted by dissolving sodium bicarbonate. A [DIC] of 7 mM was selected based on a previous study that described the effect of the gaseous CO$_2$ concentration on the [DIC] in the liquid phase. Moreover, a nonbuffered medium was used, and this medium was prepared using the same method as that used for MTP except for the addition of buffer materials (i.e. MES and Tris base). The pH was then autonomously adjusted to a value of 7.0. The specific growth rate of microalgae ($\mu$; d$^{-1}$) was evaluated according to the following Eq. (1):

$$\mu = (\ln OD_{800,f} - \ln OD_{800,i})/(t_f - t_i)$$  

where OD$_{800,f}$ and OD$_{800,i}$ are the final and initial optical densities at 800 nm measured at two specific time points, namely, $t_f$ and $t_i$, respectively.
RNA preparation and RNA-seq analysis. To identify high CO₂ and low pH-sensitive genes, RNA samples from *Chlamydomonas reinhardtii* grown under specific CO₂ (20% CO₂) and pH (pH 4.3) conditions that suppress the growth rate of algal term by 50% were compared with that observed under ambient control conditions (atmospheric pH 7.0) (the effective concentrations that caused a decrease in the growth rate; EC₅₀) were isolated (Fig. 1a). Prior to culture, the EC₅₀ values of CO₂ and pH were predetermined (Supplementary Fig. 1). After one day of cell growth—the exposure period was determined by repeated several times, and after the stress stimulus, the cells were shifted to high CO₂ treatments and gene annotations presented in the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Individual genes related to CO₂ tolerance (Fig. 1d and Supplementary Data 2) were selected using the topGO statistical method. Using the DEG gene sets, the R package topGO and Gene Ontology (GO) terms were assigned using the Biological Process (BP), Molecular Function (MF), and Cellular Component (CC) annotations provided in the *Chlamydomonas reinhardtii* v5.6 database. Then, their expression patterns were profiled to comprehensively assess the effect of high CO₂ conditions on the cell at the transcriptional level.

**Generation of the PMA over-expressing line.** The coding sequence of PMA4, which is a well-studied PMA, from *N. plumaginifolia* was obtained from the National Center for Biotechnology Information (NCBI, USA) database. The coding region of a C-terminal autoinhibitory domain within the original sequence (100 amino acids, 300 bp) that may reduce H⁺ efflux was deleted to confer an improved H⁺ extrusion ability to the engineered algal strain 

C. reinhardtii

(Chlamydomonas reinhardtii

fl). Using GeneOptimizer software (Thermo Fisher Scientific, USA) considering the codon usage preference of

C. reinhardtii

nuclei and synthesized using GeneArt (Supplementary Figs. 8–h). Subsequently, the gene cassette was digested with EcoRI and BgII and cloned into the pChlamy_4 protein expression vector (Thermo Fisher Scientific, USA) containing a constitutive promoter (HphB7A-Rbc S2) and Zeocin resistance gene (Sh Ble). The PMA4ACtcr-V-bearing plasmid was then linearized by Sccl prior to transformation. All restriction enzymes and T4 DNA ligase used in the cloning step were purchased from Thermo Fisher Scientific (USA). To fabricate the mVenus coding insert (Supplementary Fig. 8d), the corresponding sequence (i.e. PMA4ACtcr-V with a linker sequence) was cloned into the same expression vector backbone. The engineered

C. reinhardtii

strain was transformed by electroporation (GeneOptimizer

C. reinhardtii

ΔCter-V without PMA4

ΔCter-V-bearing plasmid was then linearized by

ScaI considering the codon usage preference of

C. reinhardtii

nuclei and synthesized using GeneArt

C. reinhardtii

ΔCter-V) was digested with a Gly-rich GGSGGGSG linker to enable the localization of the exogenous protein, and the sequence of the final expression target PMA4ACtcr-V was eventually identified (Fig. 3a). The chimeric protein-coding annotations provided in the *Chlamydomonas reinhardtii* v5.6 database. Then, their expression patterns were profiled to comprehensively assess the effect of high CO₂ conditions on the cell at the transcriptional level.

**Cell tolerance test.** The pH values of the acidic media used in the low pH stress tests were titrated with HCl (both MTPA and MTP) media. For the spot plating culture, liquid media at different pH values (i.e. pH 7.0 and pH 5.0) were supplemented with plant agar (1.5%) and 0.005 mM after autoclaving. Starting from 5 x 10^4 cells mL⁻¹, 4 serial tenfold dilutions were performed and 20 μL aliquots of each dilution were then spotted on the pH-adjusted plate. Moreover, a CO₂ tolerance test with an extremely high CO₂ condition (20%-enriched air) was conducted with the autotrophic medium (i.e. MTP with an initial pH value of 7.6) to decouple the additional acidification effect, which may be caused by the organic carbon source (i.e. acetic acid).

In addition, to exclude the adaptation effect, the cells were abruptly transferred from ambient conditions to high CO₂ conditions. The optical density (OD₅₆₀) of the cell suspension was converted into the biomass concentration (g L⁻¹) using the relevant calibration functions plotted for each strain (Supplementary Fig. 12). Linear regression curves were derived based on the correlation between the cell culture dry cell weight (DCW) and turbidity. The DCW was measured via a filter-based gravimetric method using a Whatman GE/C glass microfiber filter (GE Healthcare, USA). The cells were harvested over the course of the autotrophic cultures (at atmospheric pH 7.0 condition) of each cell line, which were separately conducted to establish the correlation. Aliquots of 100 μL and 5 μL from each culture were sampled to measure the turbidity and DW, respectively. The biomass productivity (P, g L⁻¹ d⁻¹) was then estimated using the following Eq. (2):

\[ P = (X₀ − X₁)/t₂ − t₁ \]

where \( X₀ \) (g L⁻¹) and \( X₁ \) (g L⁻¹) indicate the final and initial DCW measured at two specific time points, namely, \( t₁ \) and \( t₂ \). Given the carbon balance under autotrophic conditions, the CO₂ fixation rate (\( R_{CO₂}; \text{g CO}_₂ \text{ L}⁻₁ \text{d}⁻¹ \)) was evaluated using the following Eq. (3):

\[ CO₂ \text{ fixation rate} = \frac{C_{CO₂} − \frac{M_{CO₂}}{M_p} \times \frac{P}{M_p} \times CO₂ \text{ emission}}{C_{CO₂} \text{ emission}} \]

where \( C_{CO₂} \) and \( M_{CO₂} \) denote the carbon content (% w/w), a molecular weight of CO₂ (44.01 g mol⁻¹), and atomic mass of carbon (12.01 g mol⁻¹), respectively. In the lab-scale tolerance test, the carbon content of each cell line was assumed to be 50%. To accurately estimate the practical carbon fixation ability of the algal cells in the proof-of-concept outdoor cultivation, the carbon content of the microalgae pellet was actually determined using a 5E-CHN2200 elemental analyzer (Changsha Kaiyuan Instruments, China).

**Outdoor cultivation with coal combustion flue gas injection.** The microalgal CO₂ conversion plant for cultivating the engineered mutant (PMA4ACtcr-V4) was constructed and operated under the permission of the Korean government (license number: LML 19-912). In the demonstration plant (Fig. 4a), a proof-of-concept outdoor cultivation was implemented with a 5.33 x 10^-3 m² bubble column PBR and an autotrophic medium previously optimized for outdoor microalgal cultivation. Prior to microalgal cell inoculation, the culture system, including the PBR and 10 L of industrial water, was disinfected with high alkalinity (≈pH 12; by supplementing 10 mM KOH), and the flue gas stream emitted from the neighbor coal-fired power plant was continuously diverted into the water phase at an aerating rate of 0.1 vvm. The exhaust gas consisted of 80.57% N₂, 13.18% CO₂, 7.46% O₂, 20.27 ppm NO₂, and 32.04 ppm SO₂. Because of the interaction of
KOH and acidic components in the flue gas (e.g. CO₂, NOₓ, and SO₂), gas injection resulted in the neutralization of the aqueous phase (pH 7.1) while automatically forming a bicarbonate buffer system that is suitable for large-scale outdoor cultivation⁶⁰⁷⁰. The culture medium was then prepared by adding 7 mM NH₄Cl, 0.83 mM MgSO₄·7H₂O, 0.45 mM CaCl₂·2H₂O, 1.65 mM KH₂PO₄, 1.05 mM KH₂PO₄, 0.134 mM Na₂EDTA·2H₂O, 0.136 mM ZnSO₄·7H₂O, 0.184 mM H₃BO₃, 0.40 mM MnCl₂·4H₂O, 32.9 μM FeCl₃·7H₂O, 12.3 μM CaCl₂·6H₂O, 10 μM CuSO₄·5H₂O, and 4.44 μM (NH₄)₆MoO₃·4H₂O. Based on the autotrophic culture broth, cultivation was initiated by the inoculation of 50 mg mL⁻¹ freshly and atmospherically grows small algal seeds and was continued for two weeks (14 days). The pH of the algal culture was maintained at 6–5.6 throughout the operation (Supplementary Table 4). The resulting biomass was harvested by a continuous flow centrifuge (at 21,000 × g) and lyophilized (at a pressure of 1 kPa and a temperature of −55°C) for further analyses.

Analytical methods (total lipid content, total FAME content, and calorific value). Using the harvested biomass from the outdoor cultivation, the lipid productivity (mg lipid L⁻¹ d⁻¹), biodiesel productivity (mg FAME L⁻¹ d⁻¹), and calorific productivity (kJ L⁻¹ d⁻¹) for each strain were estimated using the following Eqs. (4)-(6):

\[
\text{Lipid productivity} = \frac{\text{Total lipid content} \times \text{v}}{\text{P}} \quad (4)
\]

\[
\text{Biodiesel productivity} = \frac{\text{Total FAME content} \times \text{v}}{\text{P}} \quad (5)
\]

\[
\text{Calorific productivity} = \frac{\text{LHV} \times \text{P}}{\text{v}} \quad (6)
\]

Where P and LHV denote the biomass productivity (mg biomass L⁻¹ d⁻¹) and the calorific value (in LHV) of the biomass, respectively. To calculate the various metrics, the P (mg biomass L⁻¹ d⁻¹), total lipid content (% w/w), total FAME content (% w/w), and LHV (kcal g⁻¹) values were determined. P was estimated following Eq. (2). To determine the total lipid content in the biomass, the lipid fraction in the dried biomass was extracted according to a modified Bligh-Dyer method and the content was determined using a gravimetric assay. For the biodiesel content analysis, FAMEs (commonly referred to as biodiesel fractions) were prepared from the remaining total lipid fraction and analyzed using an Agilent 7890A gas chromatography (GC) system (Agilent Technology, USA) equipped with a DB-23 GC column and flame ionization detector (FID). The extracted lipid sample was spiked with 10 mg of pentadecanoic acid (C15:0) as an internal standard and then converted into FAMEs through transesterification using 3% methanolic sulfuric acid. The resulting converted lipids were then quantified by GC. The Supelco® 3% methanolic sulfuric acid. The resulting converted lipids were then quantified by GC. The Supelco® 3% methanolic sulfuric acid. The resulting converted lipids were then quantified by GC. The Supelco® 3% methanolic sulfuric acid. The resulting converted lipids were then quantified by GC.

Statistics and reproducibility. The replicates of all experiments were presented in the corresponding figure legends. The results are represented as the mean values and error bars, which show the standard deviations of the mean (mean ± SD). Where necessary, a two-tailed Student’s t-test was conducted to determine whether statistically significant differences existed between the mean values of the two groups. In the analysis of RNA-seq data, the genes with \(|\log_{2}\text{FC}|>1\) and \(|\log_{2}\text{FC}|<0.50\) were considered DEGs. The statistically significant differences were determined by the generalized linear model (GLM) likelihood ratio (LR) test. Overrepresented GO terms were determined based on a cutoff value of \(p<0.05\) using Fisher's exact test method. The \(p\) values, which are the engineered strains and WT control strain were conducted in triplicate. For all experiments, the replicates of all experiments were indicated in the figure legends. The results are represented as the mean values ± standard deviation (mean ± SD).

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Chlamydomonas reinhardtii v5.6 database [https://data.igi.joe.gov/redirect/download?phytotype=organism=C. reinhardtii&expanded=281]. All sequence reads for the insertion site were deposited in the NCBI SRA with BioProject accession code PRJNA67110. Source data are provided with this paper.

Data availability

Data supporting the findings of this work are available within the paper and its Supplementary Information files. A reporting summary for this article is available as a Supplementary Information file. All sequence reads for the RNA-seq were deposited in the NCBI Sequence Read Archive (SRA) with BioProject accession code PRJNA720171. The updated Chlamydomonas reinhardtii genome and gene annotation for the RNA sequencing and DNA insertion analysis were downloaded from JGI Pythove tome v13.
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