Global demand for food and bioenergy production has increased rapidly, while the area of arable land has been declining for decades due to damage caused by erosion, pollution, sea level rise, urban development, soil salinization, and water scarcity driven by global climate change. In order to overcome this conflict, there is an urgent need to adapt conventional agriculture to water-limited and hotter conditions with plant crop systems that display higher water-use efficiency (WUE). Crassulacean acid metabolism (CAM) species have substantially higher WUE than species performing C_3 or C_4 photosynthesis. CAM plants are derived from C_3 photosynthesis ancestors. However, it is extremely unlikely that the C_3 or C_4 crop plants would evolve rapidly into CAM photosynthesis without human intervention. Currently, there is growing interest in improving WUE through transferring CAM into C_3 crops. However, engineering a major metabolic plant pathway, like CAM, is challenging and requires a comprehensive deep understanding of the enzymatic reactions and regulatory networks in both C_3 and CAM photosynthesis, as well as overcoming physiometabolic limitations such as diurnal stomatal regulation. Recent advances in CAM evolutionary genomics research, genome editing, and synthetic biology have increased the likelihood of successful acceleration of C_3-to-CAM progression. Here, we first summarize the systems biology-level understanding of the molecular processes in the CAM pathway. Then, we review the principles of CAM engineering in an evolutionary context. Lastly, we discuss the technical approaches to accelerate the C_3-to-CAM transition in plants using synthetic biology toolboxes.

1. Introduction

The global population has quadrupled over the past 100 years and will continue to increase in the 21st century [1]. To feed the growing population, crop production must increase, either by expanding the amount of agricultural land for growing crops or by increasing crop yields on existing agricultural lands. Simultaneously, ongoing and projected climate changes are (1) affecting many sectors important to society, including human health, agricultural sustainability, water supply, energy security, and food supply and (2) becoming increasingly disruptive in the coming decades [2–4]. These opposing trends are threatening our global food and energy security [5]. To meet this challenge, various approaches have been explored to increase the productivity of agricultural crops [6–9]. Among them, one of the most direct approaches is engineering crassulacean acid metabolism (CAM) into C_3 crops to enhance water-use efficiency (WUE) in plants [9] thereby allowing such crops to be grown on marginal lands with reduced fresh water inputs.
To adapt to various environments on Earth, plant species have evolved several photosynthetic chemistries—C_3, C_4, and CAM photosynthesis [10]. The way plants fix atmospheric CO_2 is the key to distinguish different photosynthesis. C_3 photosynthesis is a one-stage process that produces a three-carbon compound (3-phosphoglyceric acid) via the Calvin-Benson-Bassham (CBB) cycle, while C_4 photosynthesis and CAM photosynthesis are two-stage processes, with the first stage fixing CO_2 into a series of four-carbon compounds from oxaloacetate to malate, followed by the secondary stage, where four-carbon compounds are decarboxylated, releasing CO_2 to be refixed via the CBB cycle. In C_4 plants, photosynthesis is separated spatially (mesophyll and bundle sheath cells), whereas in CAM photosynthesis CO_2 fixation is separated temporally (day and night). In CAM plants, stomata close during part or all of the day to reduce water loss, and the CO_2 is released from the malate generated during the first CO_2-fixing stage, resulting in enhanced plant WUE in comparison with C_3 or C_4 plants. WUE is the crop’s ability to assimilate a unit of carbon per unit of water consumed [11]. However, gas exchange in the leaf to obtain CO_2 inevitably results in water loss. The CAM solution to this problem is to open the stomata at night and fix carbon into malic acid, then close the stomata during the heat of the day, and release the stored CO_2 to the CBB cycle, maximizing WUE. Typically, CAM species have very high WUE, at least six- and three-fold greater than that of C_3 and C_4 plants, respectively [12].

Fresh water is the most critical resource of sustainable agriculture, and approximately 42% of the land area on Earth is classified as dryland [13, 14], where precipitation is inadequate for major conventionally grown C_3 or C_4 crops. Bioengineering CAM into C_3 plants is a potential solution to these challenges. However, engineering a major metabolic pathway like CAM is not a trivial task. Not only does it require a deep understanding of the metabolic and regulatory pathways during CAM photosynthesis, but also it requires precise regulation of the enzymatic activities, intracellular transporters, and stomatal conductance [9, 15, 16].

CAM species have been increasingly considered important climate-resilient species in the world and are a crucial driving force of ecosystem function in arid areas [17]. Recently, important achievements were made in CAM plant genomics research, significantly increasing our knowledge on the molecular mechanisms underlying CAM photosynthesis [17–20]. However, the application of this basic knowledge to CAM engineering is still limited due to technical challenges, including the lack of robust biosystems design capabilities for reconfiguring signaling and metabolic pathways in plants. Recently, biosystems design, integration of systems biology, and synthetic biology based on genome editing have emerged as innovative approaches for genetic improvement of complex biological systems in plants, microbes, and animals [21]. And as such, opportunities for revolutionizing agriculture with synthetic biology are emerging [22].

This review is intended to inspire the utilization of a biosystems design approach to accelerate C_3-to-CAM progression. First, we provide a summary of the molecular mechanisms underpinning CAM photosynthesis based on systems biology research. Second, we discuss the principles of CAM engineering in an evolutionary context. Lastly, we integrate the capabilities of gene editing and synthetic biology for CAM engineering, with a focus on building a CAM-on-demand system to increase plant resistance to episodic or seasonal drought stress.

2. A Systems Biology-Level Understanding of CAM Photosynthesis

The exploration of the molecular mechanisms of CAM is critical for CAM engineering in C_3 plant species. CAM features four core functional modules: (1) a carboxylation module to fix CO_2 and accumulate malic acid in the vacuole during the nighttime, (2) a decarboxylation module to release CO_2 from malic acid during the daytime for refixation mediated by ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) [9, 23] (Figure 1), (3) a stomatal control module to open stomata during the night and close them during the day, and (4) an anatomical module to increase the succulence of the leaf tissue [9]. A distinctive feature of CAM plants is that the stomata in the leaves remain closed during most or all of the daytime but open during the nighttime to take up CO_2, reducing water loss and correspondingly increasing WUE due to the lower evapotranspiration rates at night. Over the past ten years, genes in these functional modules (Table 1) have been identified using systems biology approaches, which involved multiomics (e.g., genomics, transcriptomics, metabolomics, and proteomics), metabolic modeling, and molecular genetic technologies such as RNA interference (RNAi) and gene editing mediated by clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) systems.

2.1. Genes in the CAM Carboxylation Module

After atmospheric CO_2 enters the mesophyll cells, it is converted to HCO_3^- by beta-carboxic anhydrase (β-CA), which is further, in combination with phosphoenolpyruvate (PEP), converted to oxaloacetate (OAA) by PEP carboxylase (PEPC) in the cytosol [24]. In most CAM plants, the reversible phosphorylation-dephosphorylation of PEPC mediated by PEPC kinase (PPCK) and possibly protein phosphatase 2A (PP2A) is understood to be under the control of the circadian clock (Figure 1) [25]. PEPC1 and PEPC2 are two most abundant PEPC transcripts in Kalanchoe fedtschenkoi. Two different patterns of convergent evolution are understood to be relevant to the carboxylation module. In the first pattern, the shift of PPCK expression from the light period to the dark period promoted the activation of PEPC1, as revealed in K. fedtschenkoi and Ananas comosus [20, 25]. In the second pattern, a single amino acid change from an arginine (R)/lysine (K) to histidine (H) to an aspartic acid (D) residue at the 509th position counting from N-terminal occurred to keep PEPC2 active without being phosphorylated, as observed in CAM species Phalaenopsis aequustris and K. fedtschenkoi [20]. Then, NAD(P)-malate dehydrogenase (NAD(P)-MDH) converts OAA to malate, which is transported into the vacuole by an aluminum-activated malate transporter (ALMT) or a tonoplast dicarboxylate transporter (tDT) (Figure 1) [26–29]. Recently, ectopic overexpression of each of the five individual carboxylation proteins (β-CA2, NAD-MDH1, NAD-MDH2,
PEPC1, and PPCK1) from *Mesembryanthemum crystallinum*, which is a facultative CAM species, enhanced leaf growth, along with an increase in organic acid accumulation and stomatal conductance in *Arabidopsis thaliana* [30]. The increased plant size and biomass yield in the transgenic *Arabidopsis* plants might arise from the release of intracellular CO2, which reduced photorespiration and consequently promoted plant growth [30].

2.2. Genes in the CAM Decarboxylation Module. During the daytime, the malic acid is moved out of the vacuole and subsequently decarboxylated to release CO2 for Rubisco-mediated fixation in the chloroplast, generating carbohydrates through the CBB cycle (Figure 1). Two different likely species-dependent processes for malate decarboxylation occur according to whether the plants contain high levels of PEP carboxykinase (PEPCK) or NAD(P)-malic enzyme (NAD(P)-ME) (Figure 1). In the NAD(P)-ME-mediated decarboxylation process, malate is converted by NAD(P)-ME to pyruvate, along with the release of CO2 in the cytosol (or mitochondria/chloroplast), followed by subsequent conversion of pyruvate to PEP mediated by pyruvate orthophosphate dikinase (PPDK). In this process, the reversible phosphorylation-dephosphorylation of PPDK, catalyzed by the PPDK regulatory protein (PPDK-RP), results in activation-inactivation of PPDK in the light-dark cycle [31]. In the PEPCK-mediated decarboxylation process, NAD(P)-MDH converts malate to OAA, which is subsequently

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**Figure 1: A simplified view of the crassulacean acid metabolism (CAM) photosynthetic pathway including key enzymes, regulatory proteins, and transporters.**
Table 1: List of known genes within the functional CAM modules.

| Protein name | Gene locus | Definition (subcellular location) | Species | Reference |
|--------------|------------|-----------------------------------|---------|-----------|
| Carboxylation module | | | |
| β-CA | Kaladp0018s0289 | β-Type carbonic anhydrase | K. fedtschenkoi | [20] |
| β-CA2 | Mcr01099291 | β-Type carbonic anhydrase 2 (cytosol) | M. crystallinum | [30] |
| PEPC1 | Kaladp0095s0055 | Phosphoenolpyruvate carboxylase 1 | K. fedtschenkoi | [20] |
| PEPC1 | Mcr0091951 | Phosphoenolpyruvate carboxylase 1 (cytosol) | M. crystallinum | [30] |
| PEPC1 | Kalax.0018s0056.1 | | | |
| PEPC2 | Kaladp0048s0058 | Phosphoenolpyruvate carboxylase 2 | K. fedtschenkoi | [20] |
| PPCK | Kaladp0037s00517 | PEPC kinase | K. fedtschenkoi | [20] |
| PPCK1 | Mcr011042t1 | PEPC kinase 1 (cytosol) | M. crystallinum | [30] |
| NAD-MDH | Kaladp0022s0111 | NAD-malate dehydrogenase | K. fedtschenkoi | [20] |
| NAD-MDH1 | Mcr0094161 | NAD-malate dehydrogenase 1 (cytosol) | M. crystallinum | [30] |
| NAD-MDH2 | Mcr0089741 | NAD-malate dehydrogenase 2 (mitochondria) | M. crystallinum | [30] |
| NADP-MDH1 | Mcr003981 | NADP-malate dehydrogenase 1 (chloroplast) | M. crystallinum | [30] |
| ALMT6 | Kaladp0062s0038 | Tonoplast aluminum-activated malate transporter 6 | K. fedtschenkoi | [20] |
| ALMT4 | Kaladp0062s0038 | Tonoplast aluminum-activated malate transporter 4 (tonoplast membrane) | M. crystallinum | Lim et al., unpublished data |
| tDT | | Tonoplast dicarboxylate transporter | Agave | [37] |
| tDT | | Tonoplast dicarboxylate transporter (tonoplast membrane) | M. crystallinum | Lim et al., unpublished data |
| Decarboxylation module | | | |
| NAD-ME | Mcr0213671 | NAD-dependent malic enzyme | K. fedtschenkoi | [20] |
| NAD-ME1 | Mcr0032671 | NAD-dependent malic enzyme 1, alpha subunit (mitochondria) | M. crystallinum | [30] |
| NAD-ME2 | Mcr0032671 | NAD-dependent malic enzyme 2, beta subunit (mitochondria) | M. crystallinum | [30] |
| NADP-ME | Kaladp0092s0166 | NADP-dependent malic enzyme | K. fedtschenkoi | [20] |
| NADP-ME1 | Mcr0032381 | NADP-dependent malic enzyme 1 (cytosol) | M. crystallinum | [30] |
| NADP-ME2 | Mcr0032381 | NADP-dependent malic enzyme 2 (chloroplast) | M. crystallinum | [30] |
| PPDK | Mcr0097361 | Pyruvate, orthophosphate dikinase (chloroplast) | M. crystallinum | [30] |
| PPDK-RP | Kaladp0010s00106 | Pyruvate, orthophosphate dikinase-regulatory protein | K. fedtschenkoi | [20] |
| PPDK-RP | Mcr0070471 | Pyruvate, orthophosphate dikinase (chloroplast) | M. crystallinum | [30] |
| PECK | | Phosphoenolpyruvate carboxykinase | K. fedtschenkoi | [20] |
| PPCK1 | AF162661 | Phosphoenolpyruvate carboxykinase | K. fedtschenkoi | [25] |
| PECK | | Phosphoenolpyruvate carboxykinase (cytosol) | M. crystallinum | [30] |
| Stomatal regulation module | | | |
| PHOT2 | Kaladp0033s00113 | Blue light receptor phototropin 2 | K. fedtschenkoi | [34] |
| AKT2 | | Arabidopsis shaker family K⁺ channels 2/3 | Agave | [37] |
| PPI | | Protein phosphatase 1 | K. pinnata, K. daigremontiana | [33] |
| PM H⁺-ATPases | | Plasma membrane H⁺-ATPase | K. pinnata, K. daigremontiana | [33] |
| Anatomical module | | | |
| VvCEB1 | | Basic helix-loop-helix transcription factor | Vitis vinifera | [59, 60] |
| PeXTH | | The xyloglucan endotransglucosylase/hydrolase | P. euphratica | [57] |
2.3. Genes Affecting Stomatal Movement. The typical gas exchange pattern in CAM plants shows extensive interspecific, intraspecific, and intraspecific variation, which complicates the study of stomatal movement. Multiple factors, including blue light, leaf-air vapor pressure deficit (VPD), leaf water status, and intercellular CO₂ concentration (Cₜ), affect the regulation of a stomatal aperture [32]. Recently, protein phosphatase 1 (PP1) and plasma membrane (PM) H⁺-ATPase were shown to play crucial roles in the blue light-dependent stomatal opening in K. daigremontiana and K. pinnata, which are two obligate CAM species [33]. Furthermore, knocking out of blue phototropin 2 (KphOT2), a light receptor, reduced stomatal conductance and Rubisco-mediated CO₂ fixation in the late afternoon when stomata are reopened and enhanced stomatal conductance and the nighttime CO₂ fixation in the CAM species K. fedtschenkoi [34]. RNAi-mediated knockdown of the CAM PEPC isozyme (PEPC1) in K. laxiflora disrupts the dark period CO₂ fixation and stomatal conductance and alters the temporal phasing of expression of genes controlling the movement of stomata, suggesting that inverse stomatal behavior is also likely to be dependent upon the activity of the primary carboxylation reaction [35]. Leaf water status usually acts on an ABA-dependent stomatal aperture in CAM plants [36–38]. Cₜ is a key driving force for CAM stomatal rhythm, which indicates the importance of metabolic control of stomatal movement in CAM plants [39, 40]. However, the key genes involved in leaf water status and Cₜ remain to be determined in CAM plants. Although the circadian oscillator can shape the rhythms of stomatal movement in CAM plants, it might not be as important as that in C₃ plants [32]. Recently, numerous candidate genes were predicted to be involved in stomatal opening and closing in CAM plants [41]. More recently, over 200 K. fedtschenkoi genes were predicted to be relevant to stomatal movement [42]. Although it would be very challenging to engineer stomatal movement, there is precedence using small molecules to control stomata [43–45]. This could be used to provide proof-of-concept studies for CAM engineering.

2.4. Genes in the Anatomical Module. Besides the critical role of temporal gene expression in CAM plants, specific functional anatomical traits are thought to be associated with optimal CAM function [46–48]. Enlarged cells allow for a larger amount of organic acids to be stored in the vacuole during the nighttime [49] and also potentially enhance water uptake and remobilization in the chlorenchyma [50]. Densely packed mesophyll cells can reduce CO₂ conductance (gₘ) within the leaf and CO₂ efflux from the leaf, increasing the capacity for performing CAM [46, 51, 52]. In typical CAM species, leaf thickness and cell size are increased whereas intracellular air space (IAS) and the length of mesophyll surface exposed to IAS per unit area (Iₘes/area) are reduced in comparison with non-CAM plant species [47]. For example, leaf thickness as a measure of tissue succulence has been associated with the performance of CAM in the Crassulaceae [53], the Orchidaceae [54], and other CAM families [47]. A comparative analysis of phylogenetically unrelated C₃+CAM and strong CAM species revealed that cell size was not related to CAM, reduced IAS and Lₘes/area were associated with CAM, and there was no difference in the proportion of IAS and Lₘes/area between strong and weak CAM species [46]. Also, a comparative analysis of multiple Clusia species ranging from C₃ to CAM with intermediate secretory tissues exposed to IAS per unit area (Iₘes/area) were associated with CAM, and there was no difference in the proportion of IAS and Lₘes/area between strong and weak CAM species [46]. For example, leaf thickness as a measure of tissue succulence has been associated with the performance of CAM in the Crassulaceae [53], the Orchidaceae [54], and other CAM families [47]. A comparative analysis of phylogenetically unrelated C₃+CAM and strong CAM species revealed that cell size was not related to CAM, reduced IAS and Lₘes/area were associated with CAM, and there was no difference in the proportion of IAS and Lₘes/area between strong and weak CAM species [46]. Also, a comparative analysis of multiple Clusia species ranging from C₃ to CAM with intermediate secretory tissues exposed to IAS per unit area (Iₘes/area) were associated with CAM, and there was no difference in the proportion of IAS and Lₘes/area between strong and weak CAM species [46]. For example, leaf thickness as a measure of tissue succulence has been associated with the performance of CAM in the Crassulaceae [53], the Orchidaceae [54], and other CAM families [47]. A comparative analysis of phylogenetically unrelated C₃+CAM and strong CAM species revealed that cell size was not related to CAM, reduced IAS and Lₘes/area were associated with CAM, and there was no difference in the proportion of IAS and Lₘes/area between strong and weak CAM species [46]. Also, a comparative analysis of multiple Clusia species ranging from C₃ to CAM with intermediate secretory tissues exposed to IAS per unit area (Iₘes/area) were associated with CAM, and there was no difference in the proportion of IAS and Lₘes/area between strong and weak CAM species [46].
vegetative and reproductive biomass, and seed yield in \textit{A. thaliana} [59]. Furthermore, overexpressing \textit{VvCEB1} was shown to increase tissue succulence and decrease intercellular air space (IAS), leading to a leaf anatomy that could potentially optimize the performance of CAM [60]. In the \textit{VvCEB1}_{opt}-overexpressing lines, the integrated and instantaneous WUE were increased, resulting in dramatically improved drought tolerance, along with enhanced salt tolerance due to a decrease in salinity uptake as well as a dilution of internal Na$^+$ and Cl$^-$ within the succulent leaves [60].

Besides the gene products involved in the above CAM modules, many other gene products are also implicated to function in CAM, such as starch phosphorylase, which is involved in the formation of PEP by glycolysis [61], and gene products involved in the regeneration of storage carbohydrates. Recently, at least 60 genes that are potentially involved in CAM evolution were identified in a comparative analysis of three obligatory CAM species (\textit{K. fedtschenkoi}, \textit{P. equestris}, and \textit{A. comosus}) and some non-CAM plant species [20] or by comparison of nonphotosynthetic and photosynthetic tissues in \textit{A. comosus} [41]. Among these genes predicted to be involved in CAM evolution, 54 genes displayed rewired diel patterns of gene expression and 6 genes showed protein sequence mutations [20]. The functional analysis of individual CAM-related genes by either overexpression [30], knockdown [25, 35, 39], or knockout [34] has laid a solid foundation for CAM biodesign. A functional CAM pathway is unlikely to result from single-gene engineering in \textit{C$_4$} plants [30]. Clearly, the engineering of multiple genes related to CAM in a modular manner is necessary to recapitulate partially or fully functional CAM modules or pathways. To move forward, the future effort for engineering CAM in \textit{C$_4$} plants should focus on the coordinated expression of the genes involved in carboxylation and decarboxylation in a manner as displayed by CAM species. Also, CAM engineering requires precise dynamic control of carbohydrate transport, degradation, and storage to supply PEP, which is required for the PEPC-mediated carboxylation process during the nighttime.

3. The Progress in the Understanding of CAM Evolution

\textit{C$_3$} photosynthesis is the predominant route that plants take in CO$_2$ and produce carbohydrates, representing approximately 95% of the Earth’s plant biomass [62]. In contrast, \textit{C$_4$} and CAM species, derived from \textit{C$_3$} ancestors, account for about 3% and 6% of flowering plant species, respectively [63, 64]. Among the angiosperms (flowering plants), \textit{C$_3$} photosynthesis has evolved independently at least 61 times in 19 families, and CAM has evolved independently in more than 400 genera across more than 38 families [15, 17, 65, 66]. Therefore, CAM photosynthesis and \textit{C$_3$} photosynthesis are thought to be the result of convergent evolution from independent \textit{C$_3$} plant lineages [15]. Among the 60 candidate genes underpinning the convergent evolution of CAM from diverse lineages of \textit{C$_3$} plants, 90% showed rewiring of diel gene expression [20]. Interestingly, all of the enzymes in CAM seem to have homologs in \textit{C$_3$} species [67, 68]. Shared biochemical properties suggest that the repeated, independent CAM and \textit{C$_4$} evolution is due to the reorganization of coopted and modified ancient metabolic pathways [69]. The involved modifications can be initiated by mutation(s) and then accommodated under selection by genomic change as the adaptive phenotype evolves [70]. Indeed, \textit{C$_4$} evolution is thought to require an enabling mutation to form an initial \textit{C$_4$} cycle, followed by selection for loss of high expression of photosynthetic genes in a certain cell type [71]. However, an enabling mutation is hypothesized not to be required for the evolution of CAM [67].

In different environments, ontogenies, and species, CAM-mediated CO$_2$ fixation accounts for $<$1% to 100% of total carbon gain [72–74]. CAM plants may be facultative (i.e., reversible induction or upregulation of the CAM pathway by environmental stress) or obligatory (i.e., mature photosynthetic tissues always perform CAM photosynthesis as a result of a preprogrammed, irreversible developmental process) [72, 74]. In addition, strong CAM and weak CAM are also widely used to define CAM species, with strong CAM meaning that ~95% of carbon intake is through the CAM pathway [72]. A recent comparative analysis of key carbon fluxes between \textit{C$_3$} and CAM pathways showed that \textit{C$_4$} plants had metabolite fluxes similar to CAM fluxes [67] (Figure 2). More recently, two alternative models have been proposed to explain the evolution of the CAM pathway [18] (Figure 3). In hypothesis 1, \textit{C$_3$} plants evolved forward to facultative CAM, weak CAM, and strong CAM in a linear manner. Under hypothesis 2, \textit{C$_3$} plants evolved into facultative CAM and weak CAM independently, and then, weak CAM further evolved into strong CAM. The \textit{C$_3$}-to-CAM continuum might explain the reversible induction of CAM by environmental stress in facultative CAM plants [67]. These hypotheses are consistent with the view that the distribution of facultative CAM is wider among vascular plants than that reported previously [72]. However, the idea of a continuum must be tempered by the evident anatomical constraints placed on the evolutionary trajectories of CAM species reflected in the bimodal distributions of \textit{C$_3$}+CAM and CAM plants revealed by large-scale $\delta^{13}$C isotopic and leaf thickness surveys [75].

Seasonal drought stress is a widely existing challenge for crop production, and this challenge could be potentially addressed through engineering a drought-inducible CAM or CAM-on-demand system [9, 30, 76]. In facultative CAM plants, CAM metabolism can be induced and reverted to the \textit{C$_3$} mode multiple times by water deficit, salinity, and high light [18, 72, 74], implying that \textit{C$_3$} photosynthesis can be engineered to be metabolically compatible with the water-use efficient adaptation. A typical CAM-on-demand system represents an engineered photosynthesis system that enables reversible CAM induction in response to drought stress (Figure 4(a)). In particular, CAM-on-demand plants would operate in the \textit{C$_3$} mode under moisture and cool conditions and temporarily switch to the CAM mode if the environment turns hot and dry. Such a system could not only possess a feature of drought tolerance under the CAM mode, but also maintain a relatively high growth rate of biomass accumulation under the \textit{C$_3$} mode, resulting
in a promising strategy in response to climate change. Therefore, from a CAM evolution-informed point of view, we can infer the following principles for CAM engineering: (1) there is no need to transfer a large number of genes from CAM species into C₃ species (it is possible that the C₃-to-CAM transition can be achieved through rewiring of temporal gene expression and rechanneling of existing metabolic flux) and (2) CAM-on-demand systems can be engineered through reversible drought-induced gene expression.

**Figure 2:** Daytime and nighttime metabolism of organic acids in C₃ and CAM plants. Arrow thickness denotes flux. Adapted from [67].

**Figure 3:** An evolution-based conceptual framework for crassulacean acid metabolism (CAM) engineering guidance. Hypothesis 1: CAM evolution followed a linear course leading from facultative CAM to strong constitutive CAM. Hypothesis 2: facultative and constitutive CAM evolved independently. The hypotheses were adapted from [18].
4. Installation of CAM-on-Demand Systems Using Gene Editing and Synthetic Biology Approaches

4.1. Genome Editing and Gene Regulation Approaches Required for CAM Engineering. There is a major difference in gene expression between facultative CAM and C₃ plants, with facultative CAM plants featuring drought-inducible expression of genes related to CAM [72]. Recently, rapid development of the CRISPR technology has provided a very powerful toolbox for basic and applied biological research. For example, the CRISPR/Cas systems can be used to generate single- or multinucleotide replacements, insertions, and deletions in the genome using CRISPR/Cas9, base editors, and prime editors and to manipulate gene expression using CRISPR activation (CRISPRa) and CRISPR interference (CRISPRi) [77–79]. These tools have been used across different species, including E. coli, yeast, human cells, and plants. CRISPRa and CRISPRi can be used for the transcriptional gene regulation to facilitate C₃-to-CAM transition, where compatible CRISPRa and CRISPRi systems promise to minimize the scale and complexity of biosystems design and engineering (Figure 3). Recently, CRISPRai was developed to simultaneously activate and inhibit gene expression in mammalian cells [80]. Developing such a CRISPRai tool in plants will clearly facilitate CAM bioengineering.

Alternately, small RNA-based RNA interference (RNAi), which regulates gene expression at the transcriptional and posttranscriptional levels, can be used together with CRISPRa for simultaneous gene activation and inhibition in plants.

Figure 4: An inducible system for C₃ crop in response to drought. (a) A CAM-on-demand system. (b) Sequence-specific transcriptional activation systems. (c) Boolean logic gates mediated CAM signaling systems. A value of 1 represents a true answer, and 0 represents a false answer.
[81, 82]. Additionally, a new gene editing method termed prime editing can perform targeted small insertions, deletions, and base swapping in a precise manner in yeast and human cells [83]. Recently, prime editing was successfully applied in plant species such as rice and wheat, although the editing efficiency is much lower than that of the mature CRISPR editing tools [84, 85]. In short, prime editing, together with base editing, will be useful for creating genomic mutations, such as single-nucleotide change and multibase substitutions (replacement, insertion, or deletion), required for C3-CAM progression [78]. Lastly, CRISPR knockout and knockin can be used for CAM engineering. Considering that the number of CAM-related genes is high, multiplex genome editing and regulation will be needed to accelerate the discovery and functional characterization of these genes, as well as to facilitate the engineering of functional CAM-related genes in C3 species. CRISPR/Cas9-enabled multiplex knockout and CRISPRa are available in plant systems [86, 87]. Engineering CAM in C3 plants will require biosystems design tools such as CRISPR-based multiplex gene editing and gene regulation, engineering of drought-responsive gene circuits, and rewiring of metabolism.

4.2. Establishing a Drought Stress Signaling Pathway. A distinguishing feature of the CAM-on-demand system is that it requires drought-inducible transcription of CAM-related genes. Specifically, CAM-on-demand will require the regulatory expression of carboxylation-related genes under drought and dark conditions, while the expression of decarboxylation-related genes will be needed under drought and light conditions. Identifying a sensor that is capable of reading multiple inputs and transmitting them to a downstream network will be indispensable. In a general context, Boolean logic gates mediate synthetic genetic circuits that can convert multiple input signals. Such circuits have been successfully implemented in various biological systems, such as yeast and mammalian cells [88, 89]. Boolean logic gates convert multiple input signals into “truth” values, where a value of 1 represents a true answer and 0 represents a false answer [90]. Typically, following a set of algorithms, these synthetic genetic circuits can generate a defined response through an integration of multiple molecular input signals [91]. A synthetic gene circuit based on an AND gate, which generates an output only when two input signals are present, can be used for drought-inducible expression of CAM-related genes. As illustrated in Figure 4(c), if inputs A and B are defined as drought and dark signals, respectively, then the downstream carboxylation-related genes cannot be activated unless both drought and dark conditions are met. It is necessary to simplify the assembly of genetic parts and lower the complexity of the intact model.

The initial target of the gene activation system will be PEP. The synthesis of PEP is indispensable in a fully functional carboxylation module because it is the key substrate for nocturnal CO2 fixation mediated by PEPC, which converges PEP and bicarbonate to OAA (Figure 1). Unlike C3 plants, in which the hydrolytic route mainly degrades starch, typical C3 CAM plants degrade starch to provide a substrate for PEP mainly through the phosphorolytic pathway [24, 61]. Conceptually, C3 plants would benefit from an engineered switch from hydrolytic to phosphorolytic starch breakdown. Specifically, during the day, the metabolic fluxes from accumulated malic acid in the vacuole towards PYR and subsequent storage carbohydrates are increased, but the flux towards sucrose or other soluble storage carbohydrates can be decreased in CAM plants (Figure 2). To achieve this feature in a C3 plant, the key enzymes and regulators, such as NAD(P)-ME, PPDK, PPDK-RP, PEPC, and ALMT, will have to be transcriptionally activated to establish the carboxylation module. To date, gene activation can be accomplished with multiple tools (as noted above) in plants, such as by using strong promoter-mediated overexpression, CRISPRa, and a TALE-mediated mTALE-Act system [87]. Among these, multiplex CRISPR-Act2.0 and mTALE-Act, which can manipulate multiple genes simultaneously, appear appropriate for this task. However, neither of these systems can activate more than four genes simultaneously based on the current technology. Therefore, a highly multiplex activation system is required to meet the needs of a fully functional carboxylation or decarboxylation module. Very recently, we developed a de novo multiplex CRISPRa system that can simultaneously perturb the expression of eight genes in A. thaliana (Yuan et al., unpublished data). Such a system is necessary to simplify the assembly of genetic parts and lower the complexity of the intact model.
In addition to the multiplex CRISPRa system, these engineered systems could be accomplished through a sequence-specific transcriptional activation system (e.g., an adapted GAL4/UAS system or an adapted TALE/RD29A system). The GAL4/UAS system, which was originally developed for studying gene expression and function in *Drosophila* [99], has become one of the most useful systems for targeted gene expression across different species. For instance, *Potri.002G146400*-encoded *PtrEPSP* was identified as a transcriptional repressor using a GAL4/UAS-mediated protoplast transient expression system in *Populus* [100]. Inspired by this work, an adapted GAL4/UAS system could be used to control carboxylation-related gene expression. This system consists of two individual parts serving for targeting and activation (Figure 4(b)). A Gal4-DNA-binding domain is fused to the transactivator VP16 (GD-VP16) to generate a transcriptional activator that targets the UAS enhancer, and GD-VP16 is driven by a tissue-specific promoter/enhancer. The other component is the carboxylation-related gene driven by a UAS enhancer. Also, the expression of GD-VP16 is regulated by an AND gate (Figure 4(c)). That is, under defined drought and dark conditions, GD-VP16 is bound to the UAS enhancer, thereby activating carboxylation-related gene expression. To provide further precision in expression, the GAL4/UAS system has been further characterized to increase the dynamic range of the system [101]. Simultaneously, an alternate independent activation system can be used to manipulate the decarboxylation-related gene expression. Here, transcriptional activator-like effectors (TALEs), containing a modular DNA-binding domain, can be used to generate chimeric transcriptional activators or repressors. A chimeric TALE-SRDX repressor can be used to repress the transcription of the transgene *RD29A::LUC* and endogenous gene *RD29A* in *A. thaliana* [102]. Again, inspired by this work, an adapted TALE/RD29A system could be used to control decarboxylation-related gene expression (Figure 4(b)). Similar to the GAL4/UAS system, one component is a TALE-DNA-binding domain-fused transactivator VP16 (TALE-VP16) driven by a tissue-specific promoter/enhancer and the other component is the decarboxylation-related gene driven by a *RD29A* promoter. The expression of TALE-VP16 is regulated by an AND gate (Figure 4(c)).

5. Iterative Design-Build-Test-Learn (DBTL) Cycles of CAM Engineering

The application of biosystems design to CAM engineering involves DBTL, which has four different phases: (I) biodesigned genetic circuits and assembly of multigene constructs, (II) delivery of biodesigned devices, (III) plant engineering, and (IV) evaluation of engineered plants (Figure 5). In phase I, synthetic devices will likely be essential components of CAM engineering. Although different synthetic switches and biosensors for controlling genome editing, gene regulation, and protein stability have already been utilized in plants, deployment of more complicated genetic circuits for genetic engineering in plants is still a big challenge [90]. This challenge is primarily caused by the experimental bottlenecks (e.g., lack of efficient plant transformation systems) and slow generation times of plants making it difficult to test the genetic circuits in plants. To overcome these limitations, protoplast-based and *Agrobacterium*-mediated leaf infiltration transient expression
assays could be used to provide a rapid and robust analysis of transgene expression and protein subcellular localization and interaction [103]. For the assembly of multigene constructs, there are multiple methods of DNA assembly available, including Gibson assembly, BioBrick assembly, Golden Gate assembly, TOPO cloning, Gateway cloning, TNT cloning, and traditional restriction enzyme cloning [16, 104–106]. Among them, the Golden Gate assembly is capable of assembling up to 24 DNA fragments in a seamless and highly efficient manner [107]. However, unexpected interactions or transcriptional interferences between neighboring transcription units in multigene constructs are commonly found in all eukaryotic organisms including plants [108, 109]. To facilitate modular construction of a CAM gene circuit composed of multiple transcription units, which must have different diel expression patterns, transcriptional interference should be avoided. To overcome this issue, genetic insulators (enhancer blocking or barrier activity) could be deployed in multigene constructs that possibly prevent these unwanted interactions and increase transgene expression in plants [110, 111].

In phase II, the conventional methods to deliver genes to plant cells can be classified into three categories: biological, physical, or chemical approaches, with the most common and preferred method being Agrobacterium-mediated plant transformation. However, to insert large constructs containing multiple genes into the plant genome with high structural and functional stability of the engineered gene modules, new methods need to be developed for multiple rounds of site-specific in planta gene stacking [112]. In phase III, tissue culture-based plant transformation is widely used to create transformed or genome-edited plants. However, creating transgenic plants through tissue culture is a bottleneck of genome editing in plants, because (1) it is only suitable for a limited number of species and genotypes, (2) it is time-consuming with low efficiency, and (3) it might cause unwanted genetic and epigenetic changes [113]. To overcome this bottleneck, two methods were recently developed for the generation of gene-edited dicotyledonous plants via de novo meristem induction by developmental regulators without in vitro culture [114]. In phase IV, robotic high-throughput phenotyping [115], in combination with omics approaches, is needed to advance functional analysis for a quick evaluation of biodesigned devices and circuitry in the transgenic or genome-edited plants. The omics-based system dynamics modeling and diel flux balance analysis [23, 116, 117] will need to be performed for reconstructions of metabolic networks to improve the performance of transgenic plants engineered with CAM. In order to optimize the biosystems design for CAM engineering, multiple iterations of the DBTL cycle will be required and possible adjustments will be made to increase precision and efficiency in each iteration.

### 6. Conclusion and Perspectives

The engineering of CAM and coadaptive traits, such as tissue succulence, holds a great potential for sustainable production of fiber, food, feed, and biofuels in water-limited areas [9, 15, 19, 60]. Initially, a deep understanding of CAM-related gene function is a key prerequisite for engineering CAM into C₃ crops [30]. Many such genes have been identified and organized into separate CAM-related modules (i.e., carboxylation, decarboxylation, and stomatal regulation). The minimum genes that are indispensable to maintain a functional module are proposed based on the knowledge of genomic research and comparative analysis. Genes that play important roles in the CAM pathway are summarized in Table 1, providing a database to guide the user in CAM engineering. Despite decades of notable progress in CAM research, a number of potentially important genes may be yet undiscovered. Comparative analysis of more CAM plant genomes will be needed to accelerate the identification of biological parts (e.g., enzymes, posttranslational modifiers, transporters, and transcription factors) for CAM engineering.

Additional research will be required to characterize the function of candidate genes inferred from the omics and comparative genomics research. Efforts will be needed to reduce the redundancy of CAM-related genes found in different CAM species. To accelerate the C₃-to-CAM engineering, wiring of appropriate temporal gene expression and rechanneling of existing metabolic flux will be essential. A CAM-on-demand system that responds to episodic or seasonal drought can be achieved in C₃ plants through reversible drought-induced gene expression to increase WUE. Considering the genes mentioned above, using single-cell technologies will enable exploration of photo-period and cell-type dynamics of CAM-related modules. By integrating with single-cell transcriptome data, the CAM modules can establish the layer of CAM regulation, which is incomplete. The regulatory network of CAM for each module can be explored by various approaches that have not been adopted to CAM research, including ATAC-Seq (Assay for Transposase-Accessible Chromatin followed by high-throughput sequencing), DAP-Seq (DNA affinity purification and sequencing), and DNase-Seq (DNase I hypersensitive site sequencing), which may provide decondensation of accessible chromatin regions that enrich motifs of transcription factors (e.g., NAC, bZIP, WRKY, NF-Y, MYB, and AP2/ERF) mentioned above [118–120].

With the nexus of new technologies like systems genetics, genome editing, synthetic biology, and gene activation systems, we are on the threshold of purposefully accelerating C₃-to-CAM progression. The CRISPR toolkit for genome editing and gene regulation provides useful tools required for CAM engineering. The engineering of synthetic circuitry in plant systems has the potential to advance our understanding and ability to manipulate genetic and metabolic networks such as CAM. The strategies for building CAM-on-demand systems are feasible using coordinated systems and synthetic biology. To achieve these goals, synthetic genetic circuits for signaling and tools for manipulating multiple gene expression simultaneously at a large scale need to be developed in plant systems. Meanwhile, some technical challenges need to be overcome. For example, the plant transformation with large-scale multigene stacking that ensures different CAM modules to be properly expressed in transgenic plants will remain a challenge for the foreseeable future. Regardless, with the effective and successful demonstrations already reported in different organisms, synthetic biosystems design
holds a great promise to enable C₃-to-CAM progression in the near future.

Disclosure

This manuscript has been authored by UT-Battelle, LLC under Contract No. DE-AC05-00OR22725 with the U.S. Department of Energy. The United States Government retains and the publisher, by accepting the article for publication, acknowledges that the United States Government retains a nonexclusive, paid-up, irrevocable, worldwide license to publish or reproduce the published form of this manuscript, or allow others to do so, for United States Government purposes. The Department of Energy will provide public access to these results of federally sponsored research in accordance with the DOE Public Access Plan (http://energy.gov/downloads/doe-public-access-plan).

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

Authors’ Contributions

XY and GY conceived the idea. GY led the writing and revision of the manuscript. MMH, DL, SDL, WCY, JCC, HL, DJW, JGC, KM, PMS, TJT, GAT, and XY contributed to the manuscript revision. All authors accepted the final version of the manuscript.

Acknowledgments

This work was supported by the Center for Bioenergy Innovation (CBI), a U.S. Department of Energy Bioenergy Research Center supported by the Office of Science Biological and Environmental Research (BER). The writing of this manuscript was also supported by the Department of Energy (Office of Science, Genomic Science Program) under award number DE-SC0008834. SDL acknowledges support from the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2019R1I1A1A01061727). DL acknowledges financial support from the National Science Foundation (NSF) under Award Number 1833402. KM acknowledges support from start-up funding provided by the University of California, Davis. PMS acknowledges support from the Department of Energy (DE-AC02-05CH11231).

References

[1] U. N. P. Division, World Population Prospects: 2019 Revision, United Nations New York, NY, 2019.
[2] E. DeNicola, O. S. Aburizaiza, A. Siddique, A. Siddique, H. Khwaja, and D. O. Carpenter, "Climate change and water scarcity: the case of Saudi Arabia," Annals of Global Health, vol. 81, no. 3, pp. 342–353, 2018.
[3] A. J. McMichael, S. Friel, A. Nyong, and C. Corvalan, "Global environmental change and health: impacts, inequalities, and the health sector," BMJ, vol. 336, no. 7637, pp. 191–194, 2008.
[4] P. Smith and P. J. Gregory, "Climate change and sustainable food production," The Proceedings of the Nutrition Society, vol. 72, no. 1, pp. 21–28, 2013.
[5] A. Karp and G. M. Richter, "Meeting the challenge of food and energy security," Journal of Experimental Botany, vol. 62, no. 10, pp. 3263–3271, 2011.
[6] A. Kubis and A. Bar-Even, "Synthetic biology approaches for improving photosynthesis," Journal of Experimental Botany, vol. 70, no. 5, pp. 1425–1433, 2019.
[7] J. R. Evans, "Improving photosynthesis," Plant Physiology, vol. 162, no. 4, pp. 1780–1793, 2013.
[8] D. C. Ducat and P. A. Silver, "Improving carbon fixation pathways," Current Opinion in Chemical Biology, vol. 16, no. 3-4, pp. 337–344, 2012.
[9] A. M. Borland, J. Hartwell, D. J. Weston et al., "Engineering cressuslacin acid metabolism to improve water-use efficiency," Trends in Plant Science, vol. 19, no. 5, pp. 327–338, 2014.
[10] J. R. Ehleringer and R. K. Monson, "Evolutionary and ecological aspects of photosynthetic pathway variation," Annual Review of Ecology and Systematics, vol. 24, no. 1, pp. 411–439, 1993.
[11] J. L. Hatfield and C. Dold, "Water-use efficiency: advances and challenges in a changing climate," Frontiers in Plant Science, vol. 10, 2019.
[12] A. M. Borland, H. Griffiths, J. Hartwell, and J. A. C. Smith, "Exploiting the potential of plants with cressuslacin acid metabolism for bioenergy production on marginal lands," Journal of Experimental Botany, vol. 60, no. 10, pp. 2879–2896, 2009.
[13] R. P. White and J. Nackoney, "Drylands, people, and ecosystem goods and services: a web-based geospatial analysis (PDF version)," World Resources Institute, 2003, January 2012, http://pdf.wri.org/drylands.pdf.
[14] M. Zika and K. H. Erb, "The global loss of net primary production resulting from human-induced soil degradation in drylands," Ecological Economics, vol. 69, no. 2, pp. 310–318, 2009.
[15] X. Yang, J. C. Cushman, A. M. Borland et al., "A roadmap for research on cressuslacin acid metabolism (CAM) to enhance sustainable food and bioenergy production in a hotter, drier world," The New Phytologist, vol. 207, no. 3, pp. 491–504, 2015.
[16] H. C. DePaoli, A. M. Borland, G. A. Tuskan, J. C. Cushman, and X. Yang, "Synthetic biology as it relates to CAM photosynthesis challenges and opportunities," Journal of Experimental Botany, vol. 65, no. 13, pp. 3381–3393, 2014.
[17] K. R. Hultine, J. C. Cushman, and D. G. Williams, "New perspectives on cressuslacin acid metabolism biology," Journal of Experimental Botany, vol. 70, no. 22, pp. 6489–6493, 2019.
[18] X. Yang, D. Liu, T. J. Tschaplinski, and G. A. Tuskan, "Comparative genomics can provide new insights into the evolutionary mechanisms and gene function in CAM plants," Journal of Experimental Botany, vol. 70, no. 22, pp. 6539–6547, 2019.
[19] N. A. Niechayev, P. N. Pereira, and J. C. Cushman, "Understanding trait diversity associated with cressuslacin acid metabolism (CAM)," Current Opinion in Plant Biology, vol. 49, pp. 74–85, 2019.
[20] X. Yang, R. Hu, H. Yin et al., "The Kalanchoë genome provides insights into convergent evolution and building blocks
of crassulacean acid metabolism,” Nature Communications, vol. 8, no. 1, p. 1899, 2017.

[21] X. Yang, L. S. Qi, A. Jaramillo, and Z. M. (M.), Cheng, “Bio-design research to advance the principles and applications of biosystems design,” BioDesign Research, vol. 2019, article 9680853, 4 pages, 2019.

[22] E. T. Wurtzel, C. E. Vickers, A. D. Hanson et al., “Revolutionizing agriculture with synthetic biology,” Nature Plants, vol. 5, no. 12, pp. 1207–1210, 2019.

[23] N. A. Owen and H. Griffiths, “A system dynamics model integrating physiology and biochemical regulation predicts extent of crassulacean acid metabolism (CAM) phases,” The New Phytologist, vol. 200, no. 4, pp. 1116–1131, 2013.

[24] S. Shameer, K. Baghalian, C. Y. M. Cheung, R. G. Ratcliffe, N. A. Owen and H. Griffiths, “Computational analysis of the productivity potential of CAM,” Nature Plants, vol. 4, no. 3, pp. 165–171, 2018.

[25] S. F. Boxall, L. V. Dever, J. Knefova, P. D. Gould, and J. Hartwell, “Phosphorylation of phosphoenolpyruvate carboxylase is essential for maximal and sustained dark CO2 fixation and core circadian clock operation in the obligate crassulacean acid metabolism species Kalanchoë fedtschenkoi,” Plant Cell, vol. 29, no. 10, pp. 2519–2536, 2017.

[26] D. B. Medeiros, K. A. Barros, J. A. S. Barros et al., “Impaired malate and fumarate accumulation due to the mutation of the tonoplast dicarboxylate transporter has little effects on stomatal behavior,” Plant Physiology, vol. 175, no. 3, pp. 1068–1081, 2017.

[27] P. Kovermann, S. Meyer, S. Hörtensteiner et al., “The Arabidopsis vacuolar malate channel is a member of the ALMT family,” The Plant Journal, vol. 52, no. 6, pp. 1169–1180, 2007.

[28] M. A. Hurth, S. J. Suh, T. Kretzschmar et al., “Impaired pH homeostasis in Arabidopsis lacking the vacuolar dicarboxylate transporter and analysis of carboxylic acid transport across the tonoplast,” Plant Physiology, vol. 137, no. 3, pp. 901–910, 2005.

[29] V. Emmerich, N. Linka, T. Reinhold et al., “The plant homolog to the human sodium/dicarboxylic cotransporter is the vacuolar malate carrier,” Proceedings of the National Academy of Sciences of the United States of America, vol. 100, no. 19, pp. 11122–11126, 2011.

[30] S. D. Lim, S. Lee, W. G. Choi, W. C. Yim, and J. C. Cushman, “Laying the foundation for crassulacean acid metabolism (CAM) biodesign: expression of the C4 metabolism cycle genes of CAM in Arabidopsis,” Frontiers in Plant Science, vol. 10, p. 101, 2019.

[31] H. M. Astle, K. Parsley, S. Aubry et al., “The pyruvate, orthophosphate dikinase regulatory proteins of Arabidopsis are both bifunctional and interact with the catalytic and nucleotide-binding domains of pyruvate, orthophosphate dikinase,” The Plant Journal, vol. 68, no. 6, pp. 1070–1080, 2011.

[32] J. Males and H. Griffiths, “Stomatal biology of CAM plants,” Plant Physiology, vol. 174, no. 2, pp. 550–560, 2017.

[33] E. Gotoh, K. Oiawato, S. I. Inoue, K. I. Shimazaki, and M. Doi, “Stomatal response to blue light in crassulacean acid metabolism plants Kalanchoe pinnata and Kalanchoe daigremontiana,” Journal of Experimental Botany, vol. 70, no. 4, pp. 1367–1374, 2019.

[34] D. Liu, M. Chen, B. Mendoza et al., “CRISPR/Cas9-mediated targeted mutagenesis for functional genomics research of crassulacean acid metabolism plants,” Journal of Experimental Botany, vol. 70, no. 22, pp. 6621–6629, 2019.

[35] S. F. Boxall, N. Kadu, L. V. Dever et al., “Kalanchoe PPC1 is essential for crassulacean acid metabolism and the regulation of core circadian clock and guard cell signaling genes,” Plant Cell, vol. 32, no. 4, pp. 1136–1160, 2020.

[36] T. Taybi and J. C. Cushman, “Abscisic acid signaling and protein synthesis requirements for phosphoenolpyruvate carboxylase transcript induction in the common ice plant,” Journal of Plant Physiology, vol. 159, no. 11, pp. 1235–1243, 2002.

[37] H. Yin, H. B. Guo, D. J. Weston et al., “Diel rewiring and positive selection of ancient plant proteins enabled evolution of CAM photosynthesis in Agave,” BMC Genomics, vol. 19, no. 1, p. 588, 2018.

[38] T. Taybi and J. C. Cushman, “Signaling events leading to crassulacean acid metabolism induction in the common ice plant,” Plant Physiology, vol. 121, no. 2, pp. 545–556, 1999.

[39] L. V. Dever, S. F. Boxall, J. Knefova, and J. Hartwell, “Transgenic perturbation of the decarboxylation phase of crassulacean acid metabolism alters physiology and metabolism but has only a small effect on growth,” Plant Physiology, vol. 167, no. 1, pp. 44–54, 2014.

[40] T. P. Wyka, H. M. Duarte, and U. E. Lüttge, “Redundancy of stomatal control for the circadian photosynthetic rhythm in Kalanchoë daigremontiana Hamet et Perrier,” Plant Biology (Stuttgart, Germany), vol. 7, no. 2, pp. 176–181, 2008.

[41] C. M. Wai, R. VanBuren, J. Zhang et al., “Temporal and spatial transcriptomic and microRNA dynamics of CAM photosynthesis in pineapple,” The Plant Journal, vol. 92, no. 1, pp. 19–30, 2017.

[42] R. C. Moseley, G. A. Tuskan, and X. Yang, “Comparative genomics analysis provides new insight into molecular basis of stomatal movement in Kalanchoë fedtschenkoi,” Frontiers in Plant Science, vol. 10, p. 292, 2019.

[43] S. Y. Park, P. Fung, N. Nishimura et al., “Abscisic acid inhibits type 2C protein phosphatases via the PYR/PYL family of START proteins,” Science, vol. 324, no. 5930, pp. 1068–1071, 2009.

[44] F. Takahashi, T. Suzuki, Y. Osakabe et al., “A small peptide modulates stomatal control via abscisic acid in long-distance signalling,” Nature, vol. 556, no. 7700, pp. 235–238, 2018.

[45] A. Ziadi, N. Uchida, H. Kato et al., “Discovery of synthetic small molecules that enhance the number of stomata: C–H functionalization chemistry for plant biology,” Chemical Communications, vol. 53, no. 69, pp. 9632–9635, 2017.

[46] E. A. Nelson and R. F. Sage, “Functional constraints of CAM leaf anatomy: tight cell packing is associated with increased CAM function across a gradient of CAM expression,” Journal of Experimental Botany, vol. 59, no. 7, pp. 1841–1850, 2008.

[47] E. A. Nelson, T. L. Sage, and R. F. Sage, “Functional leaf anatomy of plants with crassulacean acid metabolism,” Functional Plant Biology, vol. 32, no. 5, pp. 409–419, 2005.

[48] A. M. Borland, A. Leverett, N. Hurtado-Castano, R. Hu, and X. Yang, “Functional anatomical traits of the photosynthetic organs of plants with crassulacean acid metabolism,” in The Leaf: A Platform for Performing Photosynthesis, W. W. Adams III and I. Terashima, Eds., pp. 281–305, Springer International Publishing AG, 2018.

[49] U. Lüttge and P. S. Nobel, “Day-night variations in malate concentration, osmotic pressure, and hydrostatic pressure
in *Cereus validus*, *Plant Physiology*, vol. 75, no. 3, pp. 804–807, 1984.

[50] J. A. C. Smith, P. J. Schulte, and P. S. Nobel, "Water flow and water storage in *Agave deserti*: osmotic implications of crassulacean acid metabolism," *Plant, Cell & Environment*, vol. 10, no. 8, pp. 639–648, 1987.

[51] K. Maxwell, S. Caemmerer, and J. R. Evans, "Is a low internal conductance to CO$_2$ diffusion a consequence of succulence in plants with crassulacean acid metabolism?*, *Australian Journal of Plant Physiology*, vol. 24, no. 6, pp. 777–786, 1997.

[52] H. Griffiths, W. E. Robe, J. Gimus, and K. Maxwell, "Leaf succulence determines the interplay between carboxylase systems and light use during Crassulacean acid metabolism in *Kalanchoë* species," *Journal of Experimental Botany*, vol. 59, no. 7, pp. 1851–1861, 2008.

[53] M. Kluge, J. Brulfer, J. Lipp, D. Ravelomanana, and H. Ziegler, "A comparative study by $^{13}$C-analysis of crassulacean acid metabolism (CAM) in *Kalanchoë* (Crassulaceae) species of Africa and Madagascar," *Botanica Acta*, vol. 106, no. 4, pp. 320–324, 1993.

[54] K. Silvera, L. S. Santiago, and K. Winter, "Distribution of crassulacean acid metabolism in orchids of Panama: evidence of selection for weak and strong modes," *Functional Plant Biology*, vol. 32, no. 5, pp. 397–407, 2005.

[55] V. A. Barrera Zambrano, T. Lawson, E. Olmos, N. Fernandez-Garcia, and A. M. Borland, "Leaf anatomical traits which accommodate the facultative engagement of crassulacean acid metabolism in tropical trees of the genus Clusia," *Journal of Experimental Botany*, vol. 65, no. 13, pp. 3513–3523, 2014.

[56] J. M. West-Eberhard, M. N. Hsu, N. T. Kieu Nguyen et al., "CRISPRi and CRISPRa screens in mammalian cells for precision biology and medicine," *Frontiers in Plant Science*, vol. 13, no. 2, pp. 406–425, 2020.

[57] A. Bräutigam, U. Schlüter, M. Eisenhut, and U. Gowik, "On the evolutionary origin of CAM photosynthesis," *Plant Physiology*, vol. 174, no. 2, pp. 473–477, 2017.

[58] J. M. Hibberd and S. Covshoff, "The regulation of gene expression required for C4 photosynthesis," *Annual Review of Plant Biology*, vol. 61, no. 1, pp. 181–207, 2010.

[59] M. J. West-Eberhard, J. A. C. Smith, and K. Winter, "Plant science. Photosynthesis, reorganized," *Science*, vol. 332, no. 6027, pp. 311–312, 2011.

[60] M. J. West-Eberhard, *Developmental Plasticity and Evolution*, Oxford University Press, 2003.
M. W. Gander, A. Chaudhuri, M. Kamthan, and A. Datta, “Small RNAs in plants: recent development and application for crop improvement,” *Frontiers in Plant Science*, vol. 6, p. 208, 2015.

A. V. Anzalone, P. B. Randolph, J. R. Davis et al., “Search-and-replace genome editing without double-strand breaks or donor DNA,” *Nature*, vol. 576, no. 7885, pp. 149–157, 2019.

Q. Lin, Y. Zong, C. Xue et al., “Prime genome editing in rice and wheat,” *Nature Biotechnology*, vol. 38, no. 5, pp. 582–585, 2020.

H. Li, J. Li, J. Chen, L. Yan, and L. Xia, “Precise modifications of both exogenous and endogenous genes in rice by prime editing,” *Molecular Plant*, vol. 13, no. 5, pp. 671–674, 2020.

K. Xie, B. Minkenberg, and Y. Yang, “Boosting CRISPR/Cas9 multiplex editing capability with the endogenous tRNA-processing system,” *Proceedings of the National Academy of Sciences of the United States of America*, vol. 112, no. 11, pp. 3570–3575, 2015.

L. G. Lowder, J. Zhou, Y. Zhang et al., “Robust transcriptional activation in plants using multiplexed CRISPR-Act2.0 and mTALE-Act systems,” *Molecular Plant*, vol. 11, no. 2, pp. 245–256, 2018.

M. W. Gander, J. D. Vrana, W. E. Voje, J. M. Carothers, and E. Klavins, “Digital logic circuits in yeast with CRISPR-dCas9 NOR gates,” *Nature Communications*, vol. 8, no. 1, article 15459, 2017.

T. Lebar, U. Bezeljak, A. Golob et al., “A bistable genetic switch based on designable DNA-binding domains,” *Nature Communications*, vol. 5, no. 1, article 5007, 2014.

J. Andres, T. Blomeier, and M. D. Zurbriggen, “Synthetic switches and regulatory circuits in plants,” *Plant Physiology*, vol. 179, no. 3, pp. 862–884, 2019.

M. Xie and M. Fussenegger, “Designing cell function: assembly of synthetic gene circuits for cell biology applications,” *Nature Reviews Molecular Cell Biology*, vol. 19, no. 8, pp. 507–525, 2018.

A. B. Amin, K. N. Rathnayake, W. C. Yim et al., “Crassulacean acid metabolism abiotic stress-responsive transcription factors: a potential genetic engineering approach for improving crop tolerance to abiotic stress,” *Frontiers in Plant Science*, vol. 10, p. 129, 2019.

S.-Y. Park, F. C. Peterson, A. Mosquina, J. Yao, B. F. Volkman, and S. R. Cutler, “Agrochemical control of plant water use using engineered abscisic acid receptors,” *Nature*, vol. 520, no. 7548, pp. 545–548, 2015.

A. S. Vaidya, F. C. Peterson, D. Yarmolinsky et al., “A rationally designed agonist defines subfamily IIIA abscisic acid receptors as critical targets for manipulating transpiration,” *ACS Chemical Biology*, vol. 12, no. 11, pp. 2842–2848, 2017.

K. Zhang and B. Cui, “Optogenetic control of intracellular signaling pathways,” *Trends in Biotechnology*, vol. 33, no. 2, pp. 92–100, 2015.

C. Chatelle, R. Ochoa-Fernandez, R. Engesser et al., “A green-light-responsive system for the control of transgene expression in mammalian and plant cells,” *ACS Synthetic Biology*, vol. 7, no. 5, pp. 1349–1358, 2018.

K. Müller, D. Siegel, F. Rodríguez Jahnke et al., “A red light-controlled synthetic genetic expression switch for plant systems,” *Molecular BioSystems*, vol. 10, no. 7, pp. 1679–1688, 2014.

R. Ochoa-Fernandez, S. L. Samodelov, S. M. Brandl et al., “Optogenetics in plants: red/far-red light control of gene expression,” *Methods in Molecular Biology*, vol. 1408, pp. 125–139, 2016.

A. H. Brand and N. Perrimon, “Targeted gene expression as a means of altering cell fates and generating dominant phenotypes,” *Development*, vol. 118, no. 2, pp. 401–415, 1993.

M. Xie, W. Muchero, A. C. Bryan et al., “A 5-enolpyruvylshikimate 3-phosphate synthase functions as a transcriptional repressor in Populus,” *Plant Cell*, vol. 30, no. 7, pp. 1645–1660, 2018.

M. S. Belcher, K. M. Vuu, A. Zhou et al., “Design of orthogonal regulatory systems for modulating gene expression in plants,” *Nature Chemical Biology*, vol. 16, no. 8, pp. 857–865, 2020.

M. M. Mahfouz, L. Li, M. Piatek et al., “Targeted transcriptional repression using a chimeric TALE-SRDX repressor protein,” *Plant Molecular Biology*, vol. 78, no. 3, pp. 311–321, 2012.

Y. Zhang, J. Su, S. Duan et al., “A highly efficient rice green tissue protoplast system for transient gene expression and studying light/chloroplast-related processes,” *Plant Methods*, vol. 7, no. 1, p. 30, 2011.

H. C. de Paoli, G. A. Tuskan, and X. Yang, “An innovative platform for quick and flexible joining of assorted DNA fragments,” *Scientific Reports*, vol. 6, no. 1, article 19278, 2016.

R. Chao, Y. Yuan, and H. Zhao, “Recent advances in DNA assembly technologies,” *FEMS Yeast Research*, vol. 15, no. 1, pp. 1–9, 2015.

R. Xu and Q. Li, “Protocol: streamline cloning of genes into binary vectors in agrobacterium via the Gateway® TOPO vector system,” *Plant Methods*, vol. 4, no. 1, p. 4, 2008.

S. Marillonnet and R. Grützner, “Synthetic DNA assembly using Golden Gate cloning and the hierarchical modular cloning pipeline,” *Current Protocols in Molecular Biology*, vol. 130, no. 1, 2019.

K. E. Shearwin, B. P. Callen, and J. B. Egan, “Transcriptional interference—a crash course,” *Trends in Genetics*, vol. 21, no. 6, pp. 339–345, 2005.

M. Padidam and Y. Cao, “Elimination of transcriptional interference between tandem genes in plant cells,” *BioTechniques*, vol. 31, no. 2, pp. 328–334, 2001.

W. She, W. Lin, Y. Zhu et al., “The gypsy insulator of *Drosophila melanogaster*, together with its binding protein suppressor of hairy-wing, facilitate high and precise expression of transgenes in *Arabidopsis thaliana*,” *Genetics*, vol. 185, no. 4, pp. 1141–1150, 2010.

W. Jiang, L. Sun, X. Yang et al., “The effects of transcription directions of transgenes and the gypsy insulators on the transcript levels of transgenes in transgenic Arabidopsis,” *Scientific Reports*, vol. 7, no. 1, article 14757, 2017.

K. F. McCue, E. Gardner, R. Chan, R. Thilmony, and J. Thomson, “Transgene stacking in potato using the GAANTRY system,” *BMC Research Notes*, vol. 12, no. 1, p. 457, 2019.

F. Alt peter, N. M. Springer, L. E. Bartley et al., “Advancing crop transformation in the era of genome editing,” *Plant Cell*, vol. 28, no. 7, pp. 1510–1520, 2016.

M. F. Maher, R. A. Nasti, M. Vollbrecht, C. G. Starker, M. D. Clark, and D. F. Voytas, “Plant gene editing through de novo induction of meristems,” *Nature Biotechnology*, vol. 38, no. 1, pp. 84–89, 2020.
[115] J. L. Araus, S. C. Kefauver, M. Zaman-Allah, M. S. Olsen, and J. E. Cairns, “Translating high-throughput phenotyping into genetic gain,” *Trends in Plant Science*, vol. 23, no. 5, pp. 451–466, 2018.

[116] C. Y. M. Cheung, M. G. Poolman, D. A. Fell, R. G. Ratcliffe, and L. J. Sweetlove, “A diel flux balance model captures interactions between light and dark metabolism during day-night cycles in C3 and crassulacean acid metabolism leaves,” *Plant Physiology*, vol. 165, no. 2, pp. 917–929, 2014.

[117] A. M. Borland and X. Yang, “Informing the improvement and biodesign of crassulacean acid metabolism via system dynamics modelling,” *The New Phytologist*, vol. 200, no. 4, pp. 946–949, 2013.

[118] L. Song and G. E. Crawford, “DNase-seq: a high-resolution technique for mapping active gene regulatory elements across the genome from mammalian cells,” *Cold Spring Harbor Protocols*, vol. 2010, no. 2, 2010.

[119] J. D. Buenrostro, B. Wu, H. Y. Chang, and W. J. Greenleaf, “ATAC-seq: a method for assaying chromatin accessibility genome-wide,” *Current Protocols in Molecular Biology*, vol. 109, no. 1, pp. 21.29.1–21.29.9, 2015.

[120] A. Bartlett, R. C. O’Malley, S. S. C. Huang et al., “Mapping genome-wide transcription-factor binding sites using DAP-seq,” *Nature Protocols*, vol. 12, no. 8, pp. 1659–1672, 2017.