Regulation of Rubisco gene expression in C₄ plants

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Abstract

Ribulose-1,5-bisphosphate-carboxylase/oxygenase (Rubisco) incorporates inorganic carbon into an organic form, making this chloroplastic enzyme one of the most essential factors for all life on earth. Despite its central role in photosynthesis, research into regulation of the chloroplast *rbcL* and nuclear *RbcS* genes that encode this enzyme has lagged behind other plant gene systems. A major characteristic of kranz-type C₄ plants is the accumulation of Rubisco only within chloroplasts of internalized bundle sheath cells that surround the leaf vascular centers. In plants that utilize the less common single cell C₄ system, Rubisco accumulates only within one type of dimorphic chloroplasts localized to a specific region of leaf chlorenchyma cells. Understanding regulatory processes that restrict Rubisco gene expression to only one cell type or chloroplast type is a major focus of C₄ research. Regulatory steps may include transcriptional, post-transcriptional, and post-translational processes.

Rubisco and C₄ photosynthesis

Ribulose-1,5-bisphosphate-carboxylase/oxygenase (Rubisco) is found in all photosynthetic organisms [1,2,3**]. Its ability to incorporate inorganic carbon into an organic form defines it as the most basic and essential factor for maintaining life on earth. In higher plants, Rubisco's CO₂ fixation capability affects all aspects of growth and development, including viability, fecundity, stress resistance, and biomass productivity [2,4,5]. Compartmentalized within the chloroplasts of leaves and other photosynthetic tissues, it is composed of eight large (LSU, 51-58 kDa) and eight small (SSU; 12-18 kDa) subunits encoded by *rbcL* and *RbcS* genes, respectively [2,3**,5]. The chloroplast-encoded *rbcL* mRNAs are translated on prokaryotic-like ribosomes to produce the LSU. A small nuclear *RbcS* gene family encodes transcripts that are translated on cytoplasmic ribosomes to produce an SSU precursor that is targeted to chloroplasts by a transit sequence. Both subunits assemble within chloroplasts to form the L8S8 Rubisco holoenzyme. Although the two subunits are encoded and synthesized within different compartments, anterograde (nuclear to chloroplasts) and retrograde (plastid to nucleus) processes coordinate their production and synchronize their accumulation within photosynthetic cells [2,5,6**].

The assimilation of CO₂ into organic molecules is initiated when Rubisco carboxylates ribulose 1,5-bisphosphate (RuBP) to produce 2 molecules of glycerate-3-phosphate, which are then processed through the Calvin Benson cycle. The intrinsic competing oxygenase activity of this enzyme and the associated photorespiration process has led to the evolutionary development of carbon concentrating mechanisms (CCMs). These include the pyrenoid of prokaryotes, the carboxysome of algae, and the C₄ and CAM pathways of higher plants [1,2,5,7]. CCMs involve

53 numerous changes in gene expression that modify cell structure as well as alternative pathways
54 for initial carbon assimilation, partitioning, and transport. In higher plants, the C₄ pathway of
55 photosynthesis is recognized as the most common and agronomically significant CCM [8,9].

56 There are many anatomical, biochemical, and genomic variations between different C₄
57 species, reflecting multiple independent evolutionary origins for this pathway [2,5,7,8,10,11].
58 Most characteristic of all kranz-type C₄ plants is expression of *rbcL* and *RbcS* genes only within
59 the leaf bundle sheath cells (BSC), and not mesophyll cells (MC). In mature C₄ leaves,
60 specificity to BSC occurs at the levels of mRNA and protein accumulation (Graphical abstract,
61 and Figure 1). In the mature leaves of plants that utilize the less common single cell C₄ system
62 (SC-C₄), Rubisco is compartmentalized within only one type of dimorphic chloroplast that is
63 localized to a specific region of the leaf chlorenchyma cell (Figure 2) [5,12,13**].

65 **Rubisco gene expression is highly regulated in all plants**

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67 Unlike other most other signature cell-type specific C₄ photosynthesis genes that were
68 modified from a basic metabolic function in C₃ plants (such as decarboxylating malic enzymes
69 and phosphoenolpyruvate carboxylase), Rubisco functions as a photosynthetic enzyme in all
70 plants. Even with their expression modified for BSC specificity, *rbcL* and *RbcS* genes in C₄
71 plants share many common aspects with their C₃ counterparts. In all plants, Rubisco genes are
72 expressed very abundantly, producing as much as 50% of the total soluble protein in some leaf
73 cells [2,3**]. In C₄ plants, as in C₃ plants, Rubisco genes are expressed in leaves and other green
74 tissues, with little or no expression non-photosynthetic tissues such as roots. Another common
75 aspect is a requirement for illumination to activate and maintain expression. Rubisco genes are

highly responsive to developmental signals, photosynthetic metabolism, climate change, disease, and many other abiotic and biotic stresses [2,5]. All plants incorporate many levels of regulation that affect Rubisco production, from activation of transcription through the assembly, function, and degradation of the L8S8 holoenzyme. A central yet elusive question in C₄ research is what stages(s) of this complex regulatory chain have actually been modified to prevent the expression of *rbcL* and *RbcS* genes within leaf MC, while still allowing for expression and accumulation only in BSC (Figure 1, Figure 3). In addressing this question, it is also important to consider that functionally similar mechanisms and components may also ensure that Rubisco subunits accumulate only within specific chloroplast types in SC-C₄ chlorenchyma cells (Figure 2).

C₃ to C₄ transitions in Rubisco gene expression

C₄ leaf development typically involves an initial C₃-like “default” state in which *rbcL* and *RbcS* genes are expressed in both BSC and MC (or precursors) [2,5]. Transition to full C₄ Rubisco expression occurs in response to developmental or environmental signals, depending on the species. The leaves of some C₄ dicots show BSC specificity progressing in the basipetal direction as the leaves expand, with full BSC specificity initially established near the tip of young leaves and progressing toward the base. Mature leaves of these species typically show full Rubisco BSC specificity along their entire length [2,5]. This transition occurs in coordination with other changes, including cell and chloroplast morphology, vascular development, and in the case of Amaranth, the basipetal sink-source transition in photoassimilate transport. In some SC-C₄ dicot species plastid-specific partitioning of Rubisco *rbcL* mRNA and LSU protein occurs as the leaves age, in coordination with a developmental shift from

monomorphic to dimorphic chloroplast [14,15]. Unlike C₄ dicots, Rubisco mRNAs in illuminated leaves of the C₄ monocot Maize are BSC specific from their earliest detection at the leaf base. In maize, light mediates the C₃ to C₄ transition; *rbcL* and *RbcS* transcripts occur in BSC and MC of etiolated Maize, becoming BSC-specific when plants are illuminated [2,5]. In spite of underlying differences in developmental timing and leaf structure, underlying processes responsible for BSC-specific Rubisco gene expression may be conserved between these C₄ monocots and dicots. Common leaf or plastid developmental processes, together with changes in photoassimilate metabolism brought about by the sink-to-source transition (in dicots) or the initiation of photosynthesis in light (in Maize) might invoke similar signaling/regulatory mechanisms to activate BS-specific *rbcL* and *RbcS* expression within the developing leaves of many diverse C₄ plant species [2,5,7].

Levels of regulation for Rubisco gene expression in C₄ plants.

Transcriptional regulation

Several aspects of Rubisco gene expression incorporate transcriptional control. The most notable is light-regulation [2,5], where cis-acting light response elements (LREs) interact with DNA binding proteins to activate *RbcS* transcription under illumination. Such elements may have acquired dual roles for the activation of *RbcS* transcription specifically within BSC nuclei of C₄ plants. In earlier studies, maize BSC nuclei transcribed *ZmRbcS* mRNAs more abundantly than MC nuclei. Transient expression identified *ZmRbcS* noncoding regions that promoted GUS accumulation in BSC under red or blue light, but not in the dark, while other upstream or

downstream regions were shown to enable or silence *RbcS* transcription in maize BSC or MC protoplasts, respectively.

Photosynthetic genes such as *rbcL* are transcribed by the plastid encoded polymerase (PEP), and not the nuclear encoded polymerase (NEP) [5,16**]. *In organelle* run-on analysis indicated that *rbcL* transcription may contribute partially to BSC specificity, although not enough to account for specificity levels in intact C₄ plants [7]. While the ubiquitous PEP itself is likely not directly responsible for BSC-enhanced *rbcL* transcription, the possibility that associated factors could acquire C₄-related activities (such as sigma factor recognition, see [17]), is one of many questions remaining to be addressed.

While these early studies provide compelling evidence for selective or specific transcription of *rbcL* and *RbcS* in BSC, very few studies have followed up these findings to identify transcription factors or regulatory sequences involved in C₄ Rubisco transcription [2,7,18]. In contrast, transcriptional regulatory regions and potential regulatory proteins involved with C₄ expression have been identified for some other C₄ genes, including PEPCase, NAD-ME, and glycine decarboxylase [5,7,18,19].

Post-transcriptional regulation

In monocot and dicot C₄ species, control of mRNA translation and stability have been shown to regulate BSC-specific expression of both Rubisco subunit genes [2,5,7]. This post-transcriptional control is very strong, so that even high levels of constitutive overexpression in Maize could not override Rubisco compartmentalization to BSC [20]. In amaranth BSC, light-activated polysome loading and translation occurs for both *rbcL* and *RbcS* mRNAs. During

early C₄ leaf development in both amaranth and maize, Rubisco mRNAs accumulate prior to the appearance of their encoded proteins [2,5,7,11]. In fact, at an early stage of leaf development in amaranth, Rubisco mRNAs are present in both BSC and MC precursor cells, even though the Rubisco proteins accumulate only within BSC. BSC specificity for the Rubisco mRNAs occurs later, as leaf development progresses. Similarly, the lack of photosynthesis in non-green regions of *A. tricolor* leaves is correlated with loss of BSC specificity for *rbcL* and *RbcS* mRNAs, but not their encoded proteins. The lack of correlation between the initial accumulation of Rubisco mRNAs and protein in these different plants provide evidence that transcription/accumulation of *rbcL* and *RbcS* mRNAs is delayed or uncoupled from their translational activation at early stages of C₄ leaf development.

Control of transcript stability also contributes to BSC-specificity for Rubisco mRNAs. *In organello* and nuclear run-on analyses demonstrated that, although *rbcL* and *RbcS* genes are transcribed in both BSC and MC, their mRNAs accumulate only in BSC [2,5]. Furthermore, 5' and 3' UTRs from heterologous or endogenous *RbcS* mRNAs conferred partial or full BSC-specificity (respectively) to reporter genes mRNAs when transcribed constitutively in *F. bidentis* leaves. Thus, BSC specificity can be achieved even in the absence of any *RbcS* transcription elements [2,5].

Factors that affect *rbcL* and *RbcS* gene expression

It is likely that BSC-specific Rubisco accumulation integrates multiple levels of regulation (summarized in Figure 3). Some regulatory factors that have gained attention as direct or indirect regulators of Rubisco genes, and their potential involvement with BSC specificity, are summarized below.

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Golden 2 (G2) and its paralog Glk1 are transcriptional regulators that are abundantly expressed and affect overall photosynthetic development in BSC and MC, respectively. Neither are direct regulators of Rubisco gene expression [7,18].

Bundle sheath defective 2 (Bsd2) a chaperonin, binds polysome-associated *rbcL* RNA, likely functioning in the co-translational assembly of the Rubisco subunits [3**,7,18]. It is found in both BSC and MC chloroplasts.

Rubisco assembly factors 1 and 2 (Raf1, Raf2) are required for assembly of LSU and SSU subunits [3**,21]. Raf1 is BS specific in maize, and therefore could be a factor in the later (post-translational) stages of Rubisco accumulation specifically within the chloroplasts of these cells.

Scarecrow (Scr) is a transcription factor that affects BSC development in maize leaves [22]. It also affects root development, and has not been implicated in Rubisco regulation.

Rubisco LSU protein itself has an N-terminal domain that non-specifically binds RNA under oxidizing conditions [23]. It has been suggested this domain could have a role in mediating its own translational arrest, however this has not as yet been experimentally confirmed.

Mrl1 is a nuclear-encoded Pentatricopeptide repeat (PPR) protein that binds to and facilitates processing of *rbcL* mRNA in the algae *Chlamydomonas* and in the C₃ dicot *Arabidopsis* [24]. Defective processing in *Chlamydomonas* mutants inhibits *rbcL* mRNA stability, Rubisco accumulation, and photosynthesis. However *mrl1* mutants of *Arabidopsis* retain normal levels of unprocessed *rbcL* mRNA with no effect on Rubisco accumulation or photosynthetic capacity.

Rlsb is an S-1 domain RNA binding protein that selectively binds *rbcL* mRNA and co-localizes with Rubisco within chloroplasts of C₃ and C₄ plants [13**,25]. It accumulates only within BSC chloroplast in kranz species, and only within Rubisco containing central compartment chloroplasts in SC-C₄ plants. A series of biochemical and genetic studies have implicated Rlsb as a regulator of *rbcL* mRNA translation and/or stability in all plants. Its specificity to Rubisco containing chloroplasts suggests a role in cell/compartment specificity in C₄ plants. To date, this is the first regulatory protein to be implicated in the regulation of C₄ Rubisco gene expression.

Concluding remarks.

On the surface Rubisco gene expression in kranz-type C₄ plants presents a straightforward regulatory system, in which expression of *rbcL* and *RbcS* genes is maintained in the BSC, and repressed in an adjacent cell type, the MC. However, an understanding of regulatory sequences on DNA or mRNA and cis-acting factors they interact with has lagged behind other plant gene systems [5,26,27]. It is clear that Rubisco genes are highly regulated and have been modified for BSC specificity in kranz species or plastid specificity SC-C₄ plants. Identifying nucleic acid binding proteins such as RLSB will be key to understanding the

molecular basis of C₄ photosynthesis. While the mystery of Rubisco regulation in C₃ and C₄ plants is still largely unknown, innovations from molecular and biochemical analysis, systems biology, and genetics may ultimately reveal surprisingly novel mechanisms responsible for the regulation of this critical photosynthetic enzyme, providing possibilities for bioengineering C₄ traits into C₃ crops to improve productivity [28,29].

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Figure heading

Figure 1.

Localization of Rubisco large subunit (LSU) protein (top panel) and *rbcL* mRNA (bottom panel) in leaf sections prepared from mature leaves of the C₄ dicot Amaranth. Note the specific localization of the Rubisco protein and transcript to leaf BSC, with no accumulation in the MC.

For the top panel, Rubisco immunolocalization was performed using antisera specific for the Rubisco LSU, and a secondary antibody conjugated with the fluorescent dye Alexafluor 546 as described [25]. Laser scanning confocal imaging reveals Rubisco localization in this Kranz-type C₄ leaf section as yellow colored fluorescence only within the chloroplasts of BSC, and not in the chloroplasts of MC (observed as blue autofluorescence).

For the bottom panel, *in situ* hybridizations of an Amaranth leaf section was performed using using a DIG-11-UTP antisense *rbcL* probes with BM purple detection. BM Purple reaction with the hybridized *rbcL* probe was observed only in the BSC, and not the MC, indicating cell-type specific accumulation of *rbcL* mRNA in these leaves. For both panels, bar = 100 μM.

Figure 2.

Localization of Rubisco large subunit (LSU) protein to the Central Compartment Chloroplasts (CCC) in leaf cells of *Bienertia sinuspersici*, a plant that uses single cell C₄ photosynthesis. CCC, central compartment chloroplasts; PCC, peripheral compartment chloroplasts. Note that Rubisco accumulates specifically within the internalized CCCs, which are observed as a sphere of chloroplasts in the center of the cell. Little or no accumulation of Rubisco is observed within

the outermost PCCs of these cells. For this laser scanning confocal image, Rubisco is observed as a gold color, while the rest of the cell is observed by differential interfering contrast (DIC) imaging. Reaction of the *B. sinuspersici* sections with primary and secondary antisera was performed as described for Figure 1 top panel, and [13]. Scale bar = 50 μ M.

Figure 3.

Multiple stages of Rubisco gene expression and production in BSC and MC of C_4 plants. Nuclear-encoded *RbcS* genes, and genes involved in anterograde regulation of *rbcL* in the chloroplasts, are likely to be controlled at both transcriptional and post-transcriptional levels. The *rbcL* gene on the chloroplast genome is regulated primarily at post-transcriptional levels. Production stages that occur in C_4 BSC, and in all chlorenchyma cells of C_3 plants, are shown on the left. Potential regulatory points responsible for inhibiting these processes in C_4 MC are indicated by yellow crosses. This repression could be mediated by the lack of positive regulators, or possibly the presence of negative regulators that function specifically in M cells. More constitutive processes, such as nuclear export and plastid targeting are less likely to be primary C_4 regulatory points, and therefore are not shown as blocked in M cells. * indicates that *rbcL* transcription occurs in both BSC and MC chloroplasts, but could be marginally reduced in the M plastids. **1** = proposed regulatory point for the BSC-specific RLSB mRNA binding protein. **2** = production stage and potential regulatory point associated with the BSC-specific Raf1 assembly factor.

Figure 1

Figure 2

Figure 3