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Transcripts of maize *RbcS* genes accumulate differentially in C_3 and C_4 tissues

Rob M. Ewing[1,3], Gareth I. Jenkins[2] & Jane A. Langdale[1,*]
[1]*Department of Plant Sciences, University of Oxford, South Parks Road, Oxford, UK (*author for correspondence);* [2]*Plant Molecular Science Group, Division of Biochemistry and Molecular Biology, Institute of Biomedical and Life Sciences, Bower Building, University of Glasgow, Glasgow, G12 8QQ, UK;* [3]*Current address: Information Genetique & Structurale (CNRS-EP. 91), 31, Chemin Joseph Aiguier, 13402 Marseille Cedex 20, France*

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Abstract

RbcS genes exist as multigene families in most plant species examined. In this paper, we report an investigation into the expression patterns of two maize *RbcS* genes, designated in this report as *RbcS1*and *RbcS2*. We present the sequence of *RbcS2* and show that the structure of the gene has several features in common with other monocot *RbcS* genes. To determine whether *RbcS1* and *RbcS2* fulfil different functional roles with respect to the C_3 and C_4 carbon fixation pathways, we have investigated the expression patterns of the two genes in different maize tissue types. Transcripts of both genes are found at high levels specifically in bundle-sheath cells of maize seedling leaves, indicating that both genes are expressed in the C_4-type pattern. However, we show that *RbcS1* transcripts are relatively more abundant than *RbcS2* transcripts in C_3 tissues such as husk leaves. These results are discussed with respect to the evolution of C_4 carbon fixation and the mechanisms required for the cell-specific expression of *RbcS* genes.

Introduction

C_4 photosynthesis is an elaboration of the more commonly found C_3 mode of carbon assimilation, and is proposed to have evolved from C_3 photosynthesis on several independent occasions during the divergence of the flowering plants [15, 26]. In C_3 plants, CO_2 is fixed directly by ribulose bisphosphate carboxylase/oxygenase (Rubisco) in the Calvin cycle. This carboxylation reaction is inhibited when oxygen binds the active site of Rubisco [43]. To avoid the energy-wasting process of photorespiration that results from this action, C_4 plants operate an additional carbon fixation cycle which serves to concentrate CO_2 at the site of Rubisco. CO_2 is first fixed in mesophyll cells to generate a C_4 acid which is then transferred to bundle-sheath cells. Subsequent decarboxylation in the bundle-sheath releases CO_2 for refixation in the Calvin cycle [12]. The biochemical division of labour between bundle-sheath and mesophyll cells requires that photosynthetic enzymes accumulate in the appropriate cell type. Thus, in C_4 plants, the primary carboxylating enzyme, phosphoenolpyruvate carboxylase (PEP-Case), is abundant in mesophyll cells whereas Rubisco accumulates only in bundle-sheath cells.

The expression of genes encoding photosynthetic enzymes has been studied in a variety of C_4 plants. In maize, it has been shown that the expression of C_4 genes and the development of the C_4 photosynthetic system is a light-dependent process that occurs only in bundle-sheath and mesophyll cells that are immediately adjacent to a vein [19]. Accumulation of *rbcL* and *RbcS* transcripts is restricted to bundle-sheath cells in light-grown leaf tissue [17, 32, 33]. In dark-grown leaves, and in tissues such as husk leaves where meso-

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number Y09214.

phyll cells are found at a distance from a vein, transcripts encoding C_4 components such as PEPCase do not accumulate, whilst *rbcL* and *RbcS* transcripts accumulate in a non-cell-specific manner. It has been suggested, therefore, that dark-grown leaves and non-C_4 organs, such as husk leaves, adopt a default pattern of *RbcS* and *rbcL* expression, analogous to that found in leaves of C_3 plants [19].

Considerable progress has been made in elucidating the molecular mechanisms which regulate the cell-specific expression of photosynthetic enzymes in C_4 plants [3, 4, 18, 24, 25, 31, 40]. In maize, as in other higher plants, PEPCase is encoded by a multigene family [16]. A single member of the maize PEPCase gene family is expressed in a light-regulated, mesophyll cell-specific manner, whilst the other genes exhibit expression patterns consistent with non-photosynthetic function [16, 30, 39]. Promoter analysis of the maize C_4-PEPCase gene in protoplasts and in transgenic rice suggested that the primary step in the recruitment of PEPCase genes to C_4 function was the acquisition of novel *cis* elements in the promoter of the gene [24, 30].

RbcS genes also exist as small multigene families in higher-plant genomes [10]. Numerous studies have investigated the expression of *RbcS* genes in response to light or in different tissue types and shown that transcripts from individual genes accumulate differentially [10]. The molecular mechanisms governing cell-specific expression of *RbcS* genes in maize have been investigated in several recent reports [4, 28, 29, 40]. These investigations point to both transcriptional and post-transcriptional control of *RbcS* gene expression in C_4 cell types. It is not known, however, whether the products of individual *RbcS* genes are functionally divergent with respect to C_3 and C_4 carbon fixation pathways. In order to address this question, we investigated the *RbcS* gene family in maize and analysed the expression of individual genes in C_3 and C_4 maize tissues. Our results suggest that *RbcS2* is utilised to a greater extent in C_4 tissue than in C_3 tissue. We discuss the implications of these results on theories of C_4 evolution.

Materials and methods

Plant material

The maize inbred line B73 (a gift from Pioneer HiBred International) was used for all experiments. Etiolated seedlings for red-light induction experiments were grown in vermiculite in a dark-room at 28 °C. Seedlings were harvested when the 3rd leaf had just emerged (about 8 days after sowing). Light-grown seedlings were grown in a heated glasshouse (24 °C day and 20 °C night) with supplementary light from sodium lamps (16 h photoperiod, average 500 μmol m^{-2} s^{-1}). Irradiation of etiolated seedlings with monochromatic red light (λ_{max}660nm; 15 μmol m^{-2} s^{-1}) was carried out as described in a previous report [5]. Husk leaves were harvested from glasshouse-grown maize plants and were taken from 20 cm long ears.

Genomic libraries and DNA probes

RbcS1 clones were isolated from a λDash II library (*Sau*3A partial fragments from maize inbred line B73) (gift of Pamela Close, Pioneer HiBred International) using existing maize *RbcS1* genomic fragments (gift of Tim Nelson, Yale University), as probes. *RbcS2* clones were isolated from a second library (*Sau*3A partial fragments of B73 in λEmbl 3A) (Clontech, Palo Alto, CA) using a gene-specific probe, ΔSS6 (gift of Jen Sheen, Massachusetts General Hospital). The gene-specific fragments pGS1 and pGS2 were subcloned from genomic clones of *RbcS1* and *RbcS2* respectively. The cDNA clones pJL10 (*RbcS* total) and pTN1 (*Ppc1*) have been described previously [17]. The ubiquitin cDNA clone pSKUB1 was a gift from Peter Quail (USDA-PGEC, Albany) and has been described previously [9].

RNA preparation and hybridisation

RNA was extracted from maize tissue according to a previously published protocol [27], and electrophoresed, blotted and hybridised as reported previously [17]. All probes were prepared according to the random prime technique [13].

Results

Isolation and characterisation of maize RbcS *genomic clones*

In order to characterise the maize *RbcS* gene family, we sought to isolate all *RbcS* sequences present in two different maize genomic libraries (see Materials and methods). In total, 10 *RbcS* clones were isolated from these two libraries. Restriction mapping and selected sequencing showed that all 10 clones represented either

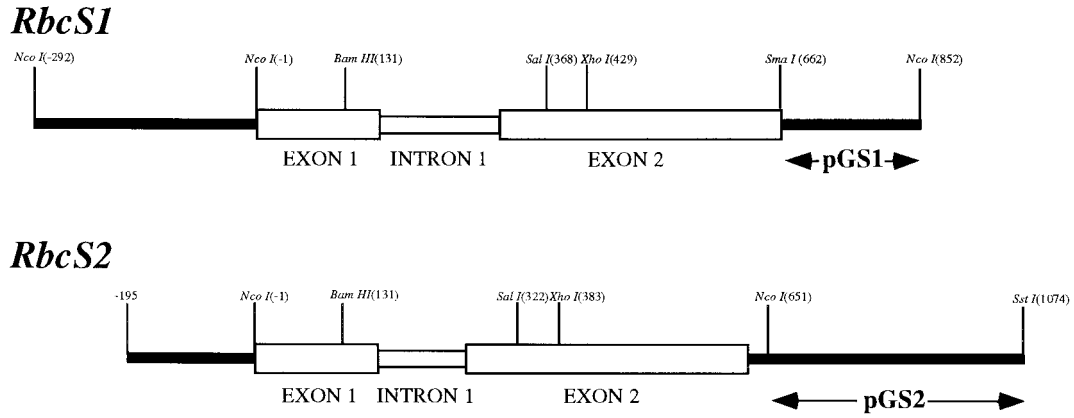

Figure 1. Schematic diagrams showing the structure of *RbcS1* and *RbcS2* genes. Exon and intron sequences are represented as shown. Subcloned fragments used as probes are shown beneath the gene structures by arrowed lines.

RbcS1 or *RbcS2* (data not shown). The structures of *RbcS1* and *RbcS2*, and the fragments used as hybridisation probes are shown schematically in Figure 1. The gene-specific probes pGS1 and pGS2, (derived from the 3′ UTR regions of *RbcS1* and *RbcS2* respectively) were used in combination with recombinant inbred (RI) lines to map *RbcS1* and *Rbcs2* to two chromosomal locations (4L1.61 and 2L095.8, respectively) [8]. No further loci were identified when RI lines were analysed with a full length cDNA clone and as such, any additional genes must map to either the *RbcS1* or *RbcS2* locus. Southern blot analyses to determine the exact number of *RbcS* genes present in the maize inbred line B73 were inconclusive. However, all of the restriction fragments analysed hybridised to either pGS1 or pGS2 and 90% of the restriction digests generated a single *RbcS1* or *RbcS2* hybridising fragment. This suggests that if additional genes are present, they are almost identical in sequence to either *RbcS1* or *RbcS2*.

The sequence of *RbcS1* genomic and cDNA clones was previously reported [20, 23]. Restriction maps of *RbcS2* genomic clones have been previously published although the sequence of the coding region, intron 1 and 3′ UTR have not been determined [29]. The complete sequence of *RbcS2* is presented in Figure 2. The structure of maize *RbcS2*, with a single intron inserted between the 2nd and 3rd codons of the mature polypeptide, is characteristic of the structure found in other grass *RbcS* genes [7, 36, 42]. In common with *RbcS* gene families of other species [10], there is a high degree of conservation between members of the maize *RbcS* gene family. The *RbcS1* and *RbcS2* coding regions differ by 21 bp over the 513 bp of exons 1 and 2. These mismatches represent 9 amino acid changes in the predicted sequences of the translation products of *RbcS1* and *RbcS2*.

RbcS1 *and* RbcS2 *are induced differentially by light*

Light inducibility of *RbcS* gene expression is a well documented phenomenon [38]. To investigate the responsiveness of individual *RbcS* genes to light, we exposed dark-grown maize seedlings to pulses of red light and analysed the accumulation of *RbcS1* and *RbcS2* transcripts at intervals thereafter. Preliminary experiments showed that etiolated seedlings exposed to a 3 min pulse of red light accumulated *RbcS* transcripts within 6 h after return to darkness (results not shown). We therefore exposed 7-day old dark-grown seedlings to a 3 min pulse of red light (15 μmol m^{-2} s^{-1}), immediately returned the seedlings to darkness, and sampled tissue (1st and 2nd leaves) at intervals up to 36 h after exposure. The results of this experiment are presented in Figure 3. The accumulation profile of *RbcS1* and *RbcS2* transcripts was broadly similar. However, there is a clear difference in fold-induction; *RbcS2* transcripts increase to higher levels (at 14 h) relative to their dark levels than do *RbcS1* transcripts. We propose therefore that *RbcS2* gene expression is more light-responsive than *RbcS1* gene expression.

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-195 CACGGAGAACGGTGGCCACCTGTCCCACATCCGCCTGTCACGCCCAACAGAGGGCGACGC

-135 GAGTCCAGCGACATGACATGGGCTCTATATATGCCGTCGGTGGGGGAGCCCCTACAGACG

-75  ACCCAAGCAAGCAAGCTCGATCTACTACTACTACTAGCTGGTACACATACTAGCCAGCCT

-15  GCCAGCCAGCTTGCCATGGCGCCCACCGTGATGATGGCCTCGTCGGCAACCGCCGTCGCC
                  M  A  P  T  V  M  M  A  S  S  A  T  A  V  A

45   CCGTTCCAGGGTCTCAAGTCCGCCGCCAGCCTCCCCGTCGCCCGCCGCAGCACCAGGAGC
     P  F  Q  G  L  K  S  A* A  S  L  P  V  A  R  R  S  T* R  S

105  CTCGGCAACGTCAGCAACGGCGGAAGGATCCGGTGCATGCAGgtaataataactcctcca
     L  G  N  V  S  N  G  G  R  I  R  C  M  Q

165 tcacatgcatcttctttgcaggctgcagctagccagtgagccagccagcaatcgagctag

225 gaactggtcgatgatgtaccatgtgtgtgcgcgcatgcagGTGTGGCCGGCCTACGGCAA
                                            V  W  P  A  Y  G  N

285 CAAGAAGTTCGAGACGCTGTCGTACCTGCCGCCGCTGTCGACGGACGACCTGCTGAAGCA
     K  K  F  E  T  L  S  Y  L  P  P  L  S  T  D  D  L  L  K  Q

345 GGTGGACTACCTGCTGCGCAACGGCTGGATACCCTGCCTCGAGTTCAGCAAGCTCGGGTT
     V  D  Y  L  L  R  N  G  W  I  P  C  L  E  F  S  K  L* G  F

405 CGTGTACCGCGAGAACTCCACCTCCCCGTGCTACTACGACGGCCGCTACTGGACCATGTG
     V  Y  R  E  N  S  T  S  P  C  Y  Y  D  G  R  Y  W  T  M  W

465 GAAGCTGCCCATGTTCGGCTGCACCGACGCCACGCAGGTGTACAAGGAGCTGCAGGAGGC
     K  L  P  M  F  G  C  T* D  A  T  Q  V  Y  K  E  L  Q  E  A

525 CATCGCCGCGTACCCGGACGCCTTCCACCGCGTCATCGGCTTCGACAACGTCAGGCAGAC
     I  A* A* Y  P  D  A  F  H  R  V  I  G  F  D  N  V* R* Q  T

585 GCAGTGCGTCAGCTTCATCGCCTACAAGCCCCCCGGCAGCGAGTAGAGACCGTGGCTAGA
     Q  C  V  S  F  I  A  Y  K  P  P  G  S  E* +

645 TCGACCCATGGCCATGCCTCTGCCTGTTGATCGGATCACCTTCTTGCATTGGTTCCTCTC

705 TCTCTCTCCCTCCTTTTTTTTCCCTTTATCTCATCATTTCTTTTCTCCTGCATGCAATGT

765 CCTTTGCTCCAACAATTCTCCTGCTGATGTATCCAGCATGGCATCATCCATCTACAATTA

825 CGTACGTAGTGCAACGACTGTCGATTCGTTGGGTGAGGAACATATATGTGAATGCAAGCT

885 CCGGCTACCATACATGTGTAATGTGTAATAGATTGATATATATACAACTGCCGAGGCGCC

945 GACAATACTTAATAACTGGTCCTTTATATATCGTCATCTGTCATTCATAACTTGTTCAGC

1005 TCACATCTACAGAAAACATGTGTTCCATTGTTATTACATGAACAGATTCTGATTCCACTA

1065 GTAGAAAAGAGCTC
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Figure 2. Sequence of *RbcS2*. The sequence is numbered with the translation start codon at position 1. The intron sequence is shown in lowercase letters and the predicted amino-acid sequence is shown below the corresponding nucleotide sequence. Amino-acids marked with an asterisk are not conserved between *RbcS1* and *RbcS2*. The stop codon is marked with a + symbol.

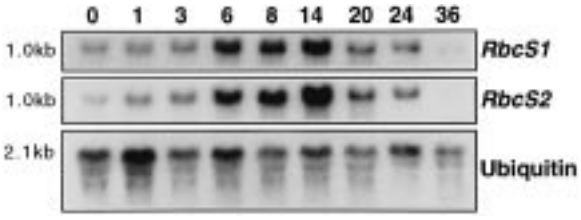

Figure 3. Time course of red light-induced expression of *RbcS1* and *RbcS2*. Numbers above the top panel are the time in hours at which tissue was sampled after light exposure. Dark-grown seedlings were exposed to a 3 min red pulse and then returned to darkness. RNA was hybridised with either pGS1 (*RbcS1*) or pGS2 (*RbcS2*) or probes. Ubiquitin hybridisations served as a loading control (bottom panel). Transcript sizes are indicated to the left of each panel.

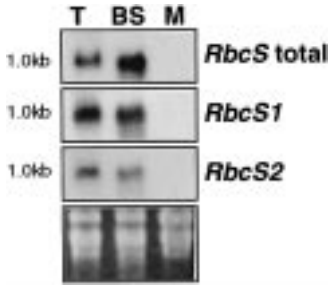

Figure 4. Bundle-sheath cell-specific expression of *RbcS1* and *RbcS2*. RNA from whole leaf (T), or separated bundle-sheath (BS) and mesophyll (M) cells hybridised with either *RbcS* cDNA probe (*RbcS* total), pGS1 (*RbcS1*) or pGS2 (*RbcS2*). Transcript sizes are indicated to the left of each panel. Equal amounts of RNA were present in each lane as judged by ethidium bromide staining of the gel (bottom panel). The BS RNA was slightly degraded after purification as indicated by the smeared hybridisation signals.

RbcS1 *and* RbcS2 *are both expressed in bundle-sheath cells of maize leaves*

In order to determine whether *RbcS1* and *RbcS2* are both expressed in bundle-sheath cells, RNA isolated from separated bundle-sheath and mesophyll cells of light-grown leaves was used in northern hybridisation experiments (Figure 4). The gene-specific fragments, pGS1 and pGS2 and a fragment from the *RbcS* coding region, pJL10, were used as probes. The results show that *RbcS1* and *RbcS2* transcripts accumulate specifically in bundle-sheath cells of light-grown maize leaves. These results are in accord with previous studies which have demonstrated the cell-specific accumulation of transcripts encoding photosynthetic components [6, 21, 32, 33].

RbcS1 *and* RbcS2 *transcripts accumulate differentially in non-C_4 tissues of maize*

We sought to investigate whether individual *RbcS* genes were expressed differentially in maize organs operating C_3 versus C_4 carbon fixation. RNA extracted from leaf blade (C_4), purified leaf blade mesophyll cells and husk leaves (C_3), was hybridised with *RbcS*, C_4 phosphoenolpyruvate carboxylase gene (*Ppc1*) and ubiquitin probes (Figure 5). In agreement with previous studies [17, 30], *Ppc1* transcripts were present in C_4 mesophyll cells and in foliar leaves operating the C_4 carbon fixation pathway, but not in C_3 tissues such as husk leaves. In contrast, *RbcS* transcripts were present both in RNA from foliar leaf tissue (C_4) and in RNA from husk leaves (C_3). Relative to their respect-

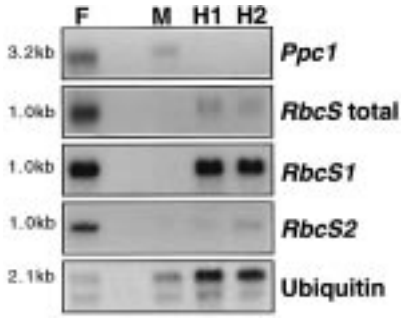

Figure 5. Expression of *RbcS1* and *RbcS2* in C_3 and C_4 maize tissue types. RNA prepared from foliar leaf blade 2 (F), isolated mesophyll cells (M), husk leaf 1 (H1) or husk leaf 2 (H2) was hybridised with the C_4 phosphoenolpyruvate carboxylase gene (*Ppc1*), *RbcS* cDNA (*RbcS* total), *RbcS1* (pGS1) or *RbcS2* (pGS2). Ubiquitin hybridisations served as a loading control (bottom panel). Transcript sizes are indicated to the left of each panel.

ive levels in foliar leaves, *RbcS1* transcripts accumulated to higher levels in husk leaves than *RbcS2* transcripts. Furthermore, in other C_3 maize organs, such as adventitious roots and prophyll, *RbcS1* transcripts were also relatively more abundant than *RbcS2* transcripts (data not shown). This suggests that transcripts in husk leaves are derived primarily from *RbcS1* and that *RbcS2* is preferentially expressed in C_4 tissue.

Discussion

We have investigated the structure and expression patterns of the two *RbcS* genes in the C_4 plant maize. RNA blot analysis of *RbcS1* and *RbcS2* transcript accumulation patterns in etiolated seedlings given a pulse of red light, showed that both genes are light-inducible. When analysed in detail, however, the profiles of accumulation of *RbcS1* and *RbcS2* transcripts are not identical; *RbcS2* transcript abundance shows a much greater-fold induction than *RbcS1*. Previous studies in other systems have demonstrated that both *RbcS1* and *RbcS2* promoter fragments fused to the reporter gene GUS direct light-regulated expression [38]. Furthermore, the *RbcS1* promoter has been shown to be less light-inducible than the *RbcS2* promoter due to the presence of a short sequence of repeated elements [29]. We propose, therefore, that the difference in steady-state levels observed in light-shifted seedlings is due to the fact the *RbcS1* promoter is not subject to the same degree of light-regulation as the *RbcS2* promoter.

Expression of *RbcS* gene family members in different plant organs, such as leaves, roots and fruits, has been investigated in several species [11, 22, 34, 35, 41]. However, no studies have documented the expression of individual *RbcS* genes in tissues operating C_3 or C_4 modes of carbon fixation. Our study demonstrates that two *RbcS* genes, both expressed abundantly in bundle-sheath cells of foliar leaves (C_4), show markedly different levels of transcript accumulation in husk leaves (C_3). Overall, *RbcS* transcripts are less abundant in husk leaf tissue than in foliar leaves. In relation to their respective levels in foliar leaves, however, *RbcS1* transcripts are more abundant in husk tissue than *RbcS2* transcripts. The differential accumulation of *RbcS1* and *RbcS2* in C_3 husk tissue might simply be a consequence of the incident light quality perceived. The arrangement of husk leaves as successive encircling layers around the ear means that incident light on inner husks is selectively depleted in red and blue wavelengths, but enriched in far-red. It has been estimated for example that second husk leaves are exposed to only 20% blue, 35% red and 70% far-red of incident light [37]. Our RNA gel blot analysis indicates that *RbcS2* is more responsive to red light than *RbcS1* expression. Selective depletion of the red and blue wavelengths incident on husk leaves might therefore selectively reduce expression of *RbcS2*. Any such effects would most likely be mediated at the post-transcriptional level since preliminary experiments suggest that *RbcS1* and *RbcS2* promoter activity in husk leaves is similar (unpublished observations).

It was previously shown that C_4 differentiation is dependent in part upon the internal structure of the organ [19]. Thus, in husk leaves in which adjacent vascular bundles are separated by 10 or more mesophyll cells, Rubisco accumulates in both mesophyll and bundle-sheath cells [1, 2, 19]. Our results suggest that individual genes comprising the maize *RbcS* gene family are not specifically utilised in C_3 or C_4 tissue. Thus, both *RbcS1* and *RbcS2* are expressed in bundle-sheath cells of foliar leaves and transcripts of both are present in husk leaf tissue. However, differential expression is observed with *RbcS2* being preferentially expressed in C_4 tissue and the non-cell-specific accumulation of *RbcS* transcripts in husk leaves being attributed primarily to *RbcS1*. These results contrast with other studies of maize C_4 gene families such as those encoding PEPCase or PPdK [14, 16, 30, 31]. In both the PEPCase and PPdK gene families, individual genes are specifically expressed in the C_4 pattern. Furthermore, transgenic experiments have shown that the maize C_4 PEPCase and PPdK genes acquired

novel 5′ *cis* elements, which direct the cell-specific, light-regulated components of their expression [24, 25]. Since transcripts of both *RbcS1* and *RbcS2* accumulate specifically in bundle-sheath cells, we suggest that both genes respond to signal(s) inducing expression in bundle-sheath cells and repression in mesophyll cells. Our results predict therefore that the molecular mechanisms that have evolved in C_4 plants to achieve bundle-sheath cell-specific expression of *RbcS* genes, do not differentiate between *RbcS1* and *RbcS2*.

Acknowledgements

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