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REVIEW

Recent progress in the single-cell C_4 photosynthesis in terrestrial plants

Shiu-Cheung LUNG[1], Makoto YANAGISAWA[2], Simon D. X. CHUONG (✉)[1]

[1] *Department of Biology, University of Waterloo, Waterloo, Ontario N2L 3G1, Canada*
[2] *Agronomy Department, Purdue University, West Lafayette, IN 47907-2054, USA*

Abstract **Currently, single-cell C_4 photosynthesis has been reported in four terrestrial plant species, *Bienertia cycloptera, B. sinuspersici, B. kavirense* and *Suaeda aralocaspica*, of family Chenopodiaceae. These species possess novel mechanisms of C_4 photosynthesis through spatial partitioning of organelles and key enzymes in distinct cytoplasmic domains within single chlorenchyma cells. Anatomical and biochemical studies have shown that the three *Bienertia* species and *S. aralocaspica* utilize biochemical and organellar compartmentation to achieve the equivalent spatial separation of Kranz anatomy but within a single photosynthetic cell. These discoveries have challenged the paradigm for C_4 photosynthesis in terrestrial plants which had suggested for more than 40 years that the Kranz feature was indispensably required for its C_4 function. In this review, we focus on the recent progress in understanding the cellular and molecular mechanisms that control the spatial relationship of organelles in these unique single-cell C_4 systems. The demonstrated interaction of dimorphic chloroplasts with microtubules and actin filaments has shed light on the importance of these cytoskeleton components in the intracellular partitioning of organelles. Future perspectives on the potential function of the cytoskeleton in targeting gene products to specific subcellular compartments are discussed.**

Keywords C_4 plants, single-cell C_4 photosynthesis, Chenopodiaceae, dimorphic chloroplasts, organelle compartmentation, photosynthetic enzymes, cytoskeleton, protein targeting

Introduction

Photosynthetic organisms assimilate atmospheric CO_2 into organic matter that is essential to all forms of life. Three modes of photosynthesis, C_3, C_4 and crassulacean acid metabolism (CAM), have been identified in terrestrial plants, and each is associated with distinct features of biochemistry, physiology and leaf anatomy (Edwards and Walker, 1983; Winter and Smith, 1996). For example, CAM plants convert atmospheric carbon into organic acids at night through the activity of phospho*enol* pyruvate carboxylase (PEPC), and the C_4 product is temporarily stored in large vacuoles in mesophyll cells for later use in the Calvin-Benson cycle during the day (Fig. 1C). These plants are succulent in their anatomy and all the photosynthetically active cells are of similar appearance and biochemical capacity with respect to carbon assimilation. On the other hand, the majority of plants perform C_3 photosynthesis where atmospheric CO_2 is assimilated directly in mesophyll cells by the key enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) into two molecules of C_3 compounds, which are then utilized for the synthesis of other products such as sucrose and starch (Fig. 1A). However, Rubisco is a bifunctional enzyme which catalyzes both carboxylation and oxygenation reactions of the same substrate, ribulose-1,5-bisphosphate (RuBP). Reaction of RuBP with CO_2 results in carbon gain whereas reaction with O_2 results in carbon loss due to a wasteful process known as photorespiration. Thus, C_3 plants generally do not perform well under extreme conditions such as drought, salinity and/or high temperature, where CO_2 becomes a limiting factor for photosynthesis (Hatch and Slack, 1970; Sage, 1999).

Contrary to the C_3 species, some plants evolved a mechanism via the C_4 dicarboxylic acid cycle for concentrating CO_2 and subsequently donating it to the C_3 pathway or Calvin-Benson cycle. These C_4 species have been identified

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Correspondence: Simon D. X. CHUONG
E-mail: schuong@uwaterloo.ca

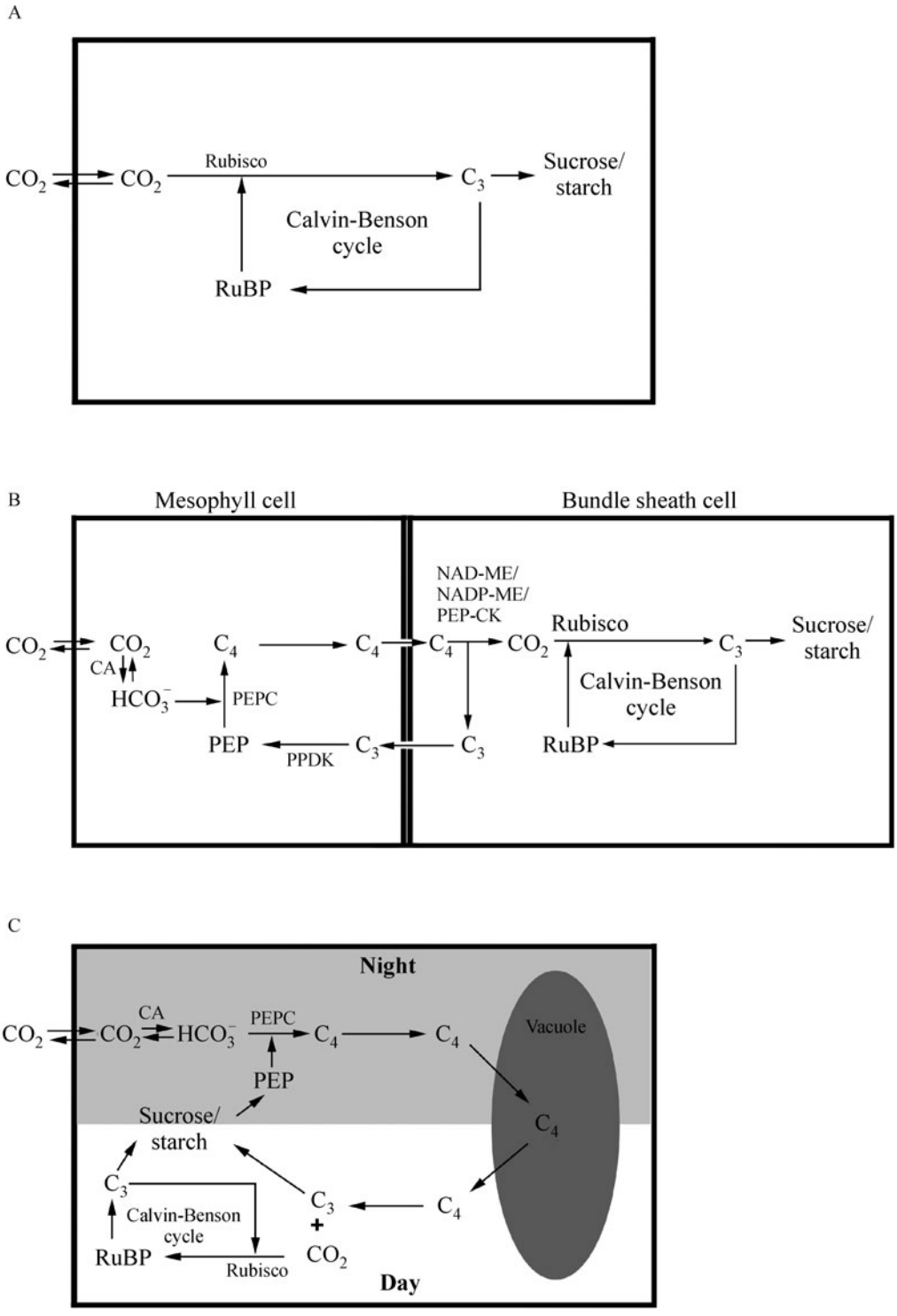

Figure 1 Simplified schematic diagrams of the C_3, C_4 and CAM photosynthetic pathways. A: In the C_3 or Calvin-Benson pathway, Rubisco catalyzes the carboxylation of CO_2 to RuBP to generate C_3 acids. Most of the C_3 acids are used for the regeneration of RuBP whereas the remainder is used in sucrose or starch synthesis. B: In the C_4 pathway, CO_2 fixation involves two cell types— the mesophyll (M) and bundle sheath (BS) cells. In the M cell, CO_2 is hydrated to HCO_3^- by carbonic anhydrase (CA) which is used to react with phosphoenolpyruvate (PEP) catalyzes by PEP carboxylase (PEPC) producing a C_4 acid. The C_4 acid is transported to the BS cell where it is decarboxylated by NAD-/NADP-malic enzyme (ME) or PEP carboxykinase (PEP-CK) yielding CO_2 and a C_3 acid (pyruvate). The CO_2 is refixed by Rubisco of the Calvin-Benson cycle while the C_3 product moves back to the M cell for the regeneration of PEP by pyruvate phosphate dikinase (PPDK). C: In the CAM pathway, CO_2 is taken up at night when stomata are open and it is hydrated by CA to HCO_3^-. PEPC catalyzes the reaction between HCO_3^- and PEP to generate a C_4 acid which is stored in the vacuole. During the day when stomata are closed, the stored C_4 acid is released from the vacuole for the decarboxylation reaction and the resulting CO_2 is converted to sucrose or starch by the Calvin-Benson cycle.

in 19 of ca. 500 families of plants including major agronomic crops such as maize, sugarcane, millets and sorghum (Sage, 2003). Terrestrial C_4 plant species generally exhibit the Kranz-type leaf anatomy consisting of two anatomically and biochemically distinct photosynthetic cell types, the mesophyll and bundle sheath cells, which function cooperatively to concentrate CO_2 at the site of Rubisco and thereby reduce photorespiration (Hatch and Slack, 1970; Edwards and Huber, 1981). In C_4 photosynthesis, atmospheric CO_2 is initially fixed into C_4 acids by PEPC in the mesophyll cells. The C_4 acids are then transported to the bundle sheath cells for the release of CO_2 by C_4 acid decarboxylases [i.e. NAD-/NADP-malic enzyme (ME) or PEP-carboxykinase (CK)] to Rubisco, the key enzyme of the Calvin-Benson cycle (Fig. 1B). The separation of different sets of biochemical reactions among the two cell types allows C_4 plants to outperform C_3 plants under CO_2-limiting conditions, as a result of higher rates of photosynthesis, and greater nitrogen and water use efficiency (Hatch and Slack, 1970; Edwards and Huber, 1981; Sage, 2003). In a Kranz-type C_4 leaf, the mesophyll cells contain two signature enzymes, PEPC for initial CO_2 fixation and pyruvate orthophosphate dikinase (PPDK) for regeneration of the PEPC substrate [i.e. phosphoenolpyruvate (PEP)], while the adjacent bundle sheath cells produce the signature decarboxylases, NAD-/NADP-ME (or PEP-CK in some species), for the liberation of CO_2 from C_4 acids and its refixation via Rubisco of the Calvin-Benson cycle (Fig. 1B).

Discovery of single-cell C_4 photosynthesis in terrestrial plants

For over 40 years, it had been generally accepted that Kranz anatomy was a required feature for C_4 photosynthesis. This paradigm has been challenged with the recent discovery that four members of the Chenopodiaceae family perform C_4 photosynthesis within individual chlorenchyma cells. What has been referred to as "single-cell C_4 photosynthesis" has been found in *Bienertia cycloptera* (Voznesenskaya et al., 2002, 2005), *B. sinuspersici* (Akhani et al., 2005; Fig. 2A-C), *B. kavirense* (Akhani et al., 2012), and *Suaeda aralocaspica* (previously called *Borszczowia aralocaspica*; Freitag and Stichler, 2000; Voznesenskaya et al., 2001; Fig. 2D–2F). The single-cell C_4 species are annual plants that are distributed in central Asia (*S. aralocaspica*) and salt deserts in countries surrounding the Persian Gulf (*B. cycloptera*; *B. sinuspersici*; *B. kavirense*). These species exhibit novel C_4 photosynthetic mechanisms through temporal expression of enzymes and

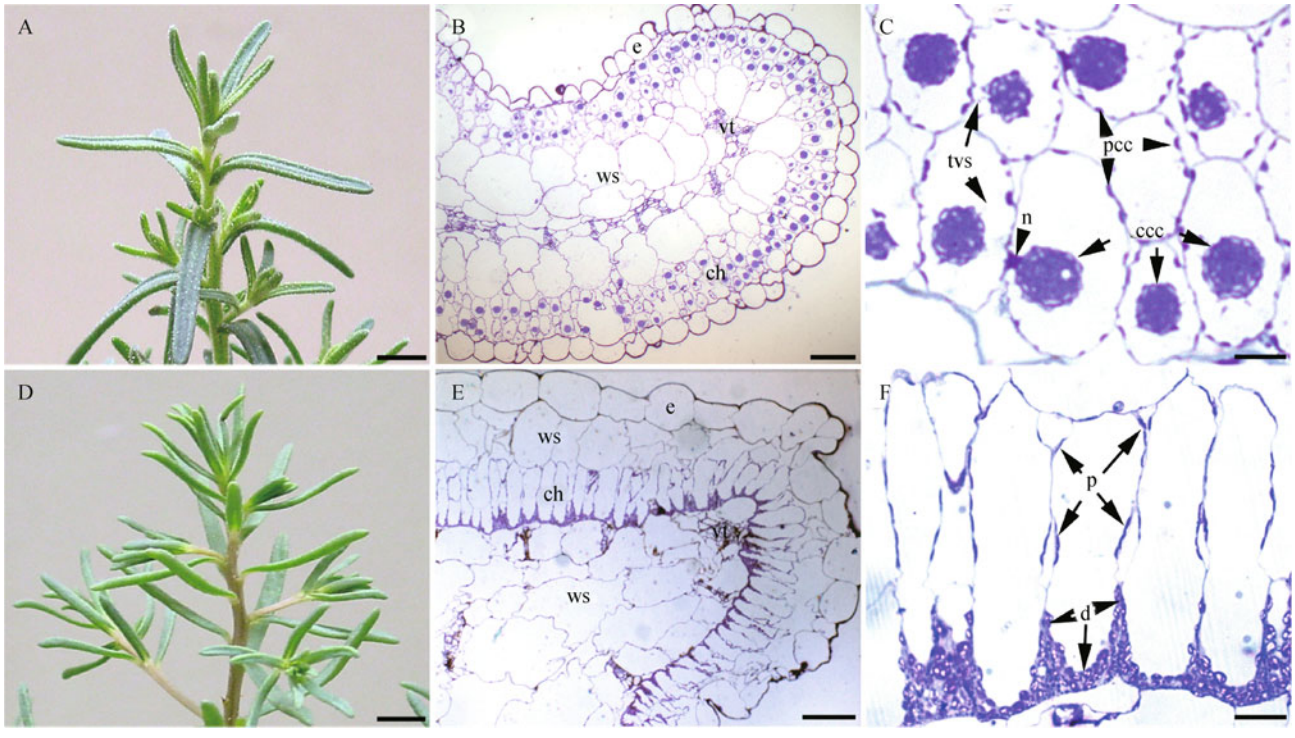

Figure 2 General leaf anatomy of *Bienertia sinuspersici* (A–C) and *Suaeda aralocaspica* (D–F). A: *B. sinuspersici* under controlled growth conditions. B: *B. sinuspersici* leaf cross section with two layers of chlorenchyma cells (ch) surrounding water storage cells (ws) and vascular tissues (vt). C: A close-up view of chlorenchyma cells showing the nucleus (n) and the central (ccc) and peripheral (pcc) cytoplasmic compartments connected by transvacuolar cytoplasmic strands (tvs; arrows). D: *S. aralocaspica* under controlled growth conditions. E: *S. aralocaspica* leaf cross section showing a single layer of elongated chlorenchyma cells sandwiched between water storage cells (ws) beneath the epidermis (e). F: A close-up view of *S. aralocaspica* chlorenchyma cells depicting the distal (d) and proximal (p) cytoplasmic compartments respectively. Scale bars (A, D) = 1 cm, (B, E) = 150 μm, (C, F) = 50 μm.

spatial partitioning of organelles into two distinct intracellular compartments within individual photosynthetic cells (Fig. 2B, E). As demonstrated by biochemical characterization of the enzymes, carbon isotope discrimination analysis and measurement of leaf gas exchange parameters, these species perform full C_4 photosynthesis in nature or under normal growth conditions. Using immunoelectron microscopy, it has been determined that this remarkable feature of C_4 photosynthesis in a single chlorenchyma cell is accomplished by partitioning of two biochemically and ultrastructurally distinct types of chloroplasts (i.e. dimorphic chloroplasts) in separate cytoplasmic compartments within the cell (Voznesenskaya et al., 2001, 2002, 2005; Chuong et al., 2006). The chlorenchyma cells of the *Bienertia* species consist of a peripheral cytoplasmic compartment (PCC) with chloroplasts containing PPDK but not Rubisco, and a central cytoplasmic compartment (CCC) with Rubisco-containing chloroplasts surrounding a large number of mitochondria containing NAD-ME (Voznesenskaya et al., 2002; Chuong et al., 2006). The two compartments are connected by thin cytoplasmic channels, which allow for the exchange of metabolic products between the two compartments (Fig. 2C). In an analogy to Kranz anatomy, the end result is equivalent to having a bundle sheath cell in the middle of a mesophyll cell with transvacuolar cytoplasmic channels connecting them.

Surprisingly, *S. aralocaspica* possesses a different mode of C_4 photosynthesis in a single cell. This species comprises of elongated palisade chlorenchyma cells, in which Rubisco-containing chloroplasts and NAD-ME-containing mitochondria are concentrated at the proximal end of the cell closer to the vascular tissues, and PPDK-containing chloroplasts are uniformly distributed at the distal end of the cell away from the vascular tissues (Voznesenskaya et al., 2001; Chuong et al., 2006; Fig. 2F). This is somewhat analogous to having the Kranz anatomy of the mesophyll and bundle sheath arrangement without the intervening cell walls. However, developmental studies demonstrated that this unique organelle partitioning was not evolved from a typical Kranz arrangement and subsequent cell wall degradation (Voznesenskaya et al., 2003). Rather, distribution of organelles at opposite ends of the chlorenchyma cell and differential expression of enzymes in the organelles at the two regions appear to occur during maturation of the *S. aralocaspica* chlorenchyma cells. In both single-cell C_4 systems, the partitioning of biochemically distinct organelles into discrete compartments results in higher CO_2 concentration around the Rubisco-containing chloroplasts, which in turn inhibits the oxygenase activity of Rubisco and photorespiration, as commonly occurs in the typical Kranz system. Thus, the partitioning of biochemistry and organelles in the single-cell C_4 systems achieves the equivalent of cellular separation of Kranz anatomy but within a single cell, as illustrated in the proposed models of single-cell C_4 photosynthesis (Edwards et al., 2004, 2008; Fig. 3).

Although the anatomy and biochemistry of these single-cell C_4 systems are slowly emerging, our overall understanding of their development is still rudimentary. The organization of organelles and biochemistry in these species represents a unique and novel system in higher plants. Furthermore, the peculiar structure and organization of these chlorenchyma cells raise questions about the developmental sequence of the mature chlorenchyma cells, about the cellular and molecular factors controlling the formation of the two intracellular compartments as a result of organelle partitioning, and about the differentiation of the dimorphic chloroplasts involving communication between the nucleus and plastids. As an initial step in further characterizing the development of the single-cell C_4 photosynthesis, anatomical and biochemical experiments were performed to examine the timing and pattern of organelle development and major photosynthesis enzyme expression in both *Bienertia* species (Voznesenskaya et al., 2005; Chuong et al., 2006; Lara et al., 2008; Park et al., 2009). It was shown that there is one common pool of plastids that partition into different subcellular compartments and then alter their biochemical expression (Voznesenskaya et al., 2005). We have also found

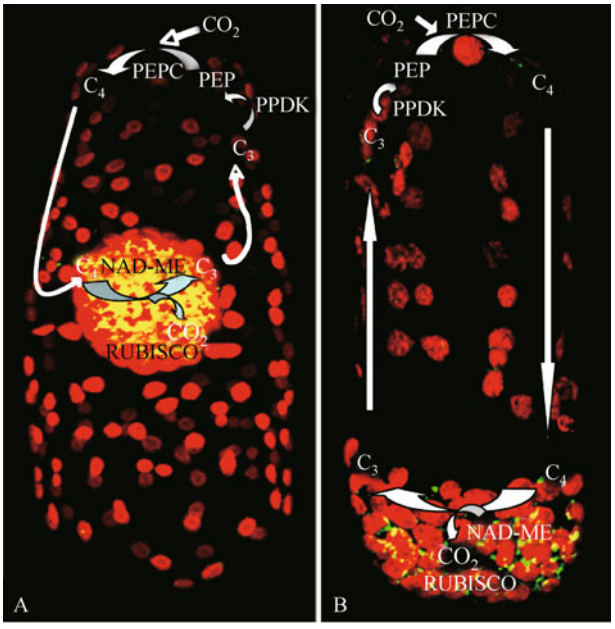

Figure 3 Proposed models for the C_4 pathway in the single-cell systems. Confocal microscopy of live *Bienertia sinuspersici* (A) and *Suaeda aralocaspica* (B) chlorenchyma cells stained with rhodamine 123 showing the partitioning of chloroplasts (red fluorescence) and mitochondria (yellow fluorescence) in distinct cytoplasmic compartments. A: In *Bienertia*, the initial fixation of atmospheric CO_2 into C_4 acids occurs in the peripheral compartment, C_4 acids diffuse via cytoplasmic channel to the central compartment for decarboxylation, and the released CO_2 are recaptured by Rubisco. B: In *S. aralocaspica*, atmospheric CO_2 enters the distal compartment of the cell where it is fixed in the C_4 cycle, the C_4 acids diffuse to the proximal compartment where they are decarboxylated, and the released CO_2 are refixed by Rubisco.

that major organelles such as chloroplasts, mitochondria and peroxisomes of the two different genera show strict partitioning into distinct compartments, a feature essential for the function of the single-cell C_4 systems (Chuong et al., 2006; Lung et al., 2011; Fig. 4). This cellular organization appears to be developmentally programmed, as chlorenchyma cells of cotyledons and dark grown leaves also showed the partitioning of organelles (unpublished data). In developing leaves of *Bienertia*, the accumulation of various proteins and transcripts was determined under different light conditions by using immunoblot and real-time PCR analyses (Lara et al., 2008). It was demonstrated that a substantial amount of Rubisco transcripts accumulates in the early stage of leaf development whereas PEPC and PPDK levels remain low until the late stage. Because the accumulation of transcripts mostly correlates to that of their proteins, it has been proposed that transcriptional control is involved in the developmental regulation of photosynthesis. Overall, these results suggest that the differentiation and compartmentation of organelles and photosynthetic enzymes are a tightly regulated and coordinated process. Recently, considerable progress has been made in the investigation of the development of the single-cell C_4 system by developing several molecular tools for analyzing gene expression. For example, procedures for isolating *Bienertia* protoplasts with intact cytoplasmic compartments have been developed and optimized allowing transient gene expression experiments to be performed in single-cell C_4 plants (Offermann et al., 2011; Lung et al., 2011; Fig. 4). To better understand the molecular basis of the single-cell C_4 system, transcriptomic and proteomic approaches were also initiated to obtain massive gene and protein profile in isolated chlorenchyma cells (Park et al., 2010). These accomplishments represent initial efforts to better understand the complex cellular organization of the single-cell C_4 species and will serve as an important tool for molecular studies examining gene expression and for the isolation of intact dimorphic chloroplasts that are essential for chloroplast preprotein import assays (Lung et al., 2012).

A role for the cytoskeleton in maintaining organelle compartmentation and possibly targeting of proteins to various subcellular compartments

Previous biochemical, anatomical and molecular studies have demonstrated that the compartmentation of major organelles such as chloroplasts, mitochondria and peroxisomes in chlorenchyma cells of *B. sinuspersici* and *S. aralocaspica* is essential for the function of the single-cell C_4 systems (Voznesenskaya et al., 2001, 2002; Chuong et al., 2006; Lara et al., 2008). Immunofluorescence microscopy has further revealed the role of the components of the cytoskeleton in the maintenance of the organelle partitioning in the *Bienertia* species and *S. aralocaspica* (Chuong et al., 2006; Park et al., 2009). It has been shown that the spatial distribution of the dimorphic chloroplasts to the CCC of *Bienertia* involved microtubules as its disruption resulted in the dispersal of chloroplasts (Chuong et al., 2006; Park et al., 2009). Previously, microtubules were also demonstrated to play a role in the anchorage of non-green plastids, the establishment of stromules in the hypocotyls of etiolated tobacco (Kwok and Hanson, 2003), as well as the positioning of mitochondria in the cortical cytoplasm (Van Gestel et al., 2002; Logan et al., 2003). On the other hand, the partitioning of organelles in *S. aralocaspica* appears to involve both actin microfilaments and microtubules since disruption of its subcellular organization was only observed in the presence of dissociating agents for both cytoskeleton components (Chuong et al. 2006). In fact, it has become apparent that actin microfilaments and microtubules interact during many developmental processes, implying a functional purpose rather than a coincidental occurrence (Chuong et al., 2005; Collings et al., 2006; Petrásek and Schwarzerová, 2009; Sampathkumar et al., 2011). This is consistent with studies demonstrating that the photo-movement and positioning of chloroplasts in plant cells are mediated by actin microfilaments and/or microtubules (Sato et al., 2001; Wada et al., 2003; Wada and Suetsugu, 2004). Thus, it is believed that the maintenance of organellar compartmentation in the single-cell C_4 species involves interactions between the dimorphic chloroplasts, with both actin microfilaments and microtubules.

Our observations of direct interaction of dimorphic chloroplasts with actin microfilaments implicate a role for actin in chloroplast movement (Fig. 4A), whereas interactions of the chloroplasts with microtubules may imply anchorage function. In *Arabidopsis*, actin microfilaments form baskets surrounding the chloroplasts (Kandasamy and Meagher, 1999). In the single-cell C_4 species, microtubules might also participate in the orderly partitioning of chloroplasts. Immunolabeling of the cytoskeleton and transient expression of fluorescent fusion proteins in protoplasts consistently revealed that, in addition to the normal cortical transverse arrangement of microtubules in the chlorenchyma cells, a portion of microtubules encircle the dimorphic chloroplasts (Chuong et al., 2006; Lung et al. 2011; Fig. 4B). Collectively, our recent data suggest that components of the cytoskeleton might mediate and maintain the organelle positioning within distinct cytoplasmic compartments in the remarkable single-cell C_4 systems.

Considering that the spatial partitioning of organelles, enzymes and metabolites is essential for C_4 photosynthesis, the structural and biochemical regulation in *Bienertia* and *S. aralocaspica* chlorenchyma cells must be complex as they are happening in the same cell. For instance, the control of organelle movement must operate under a precise and stable cellular process to establish cellular polarity. While earlier studies have indicated that the cytoskeleton, particularly microtubules, plays a crucial role in the spatial compartmentation of organelles (Chuong et al., 2006; Park et al., 2009),

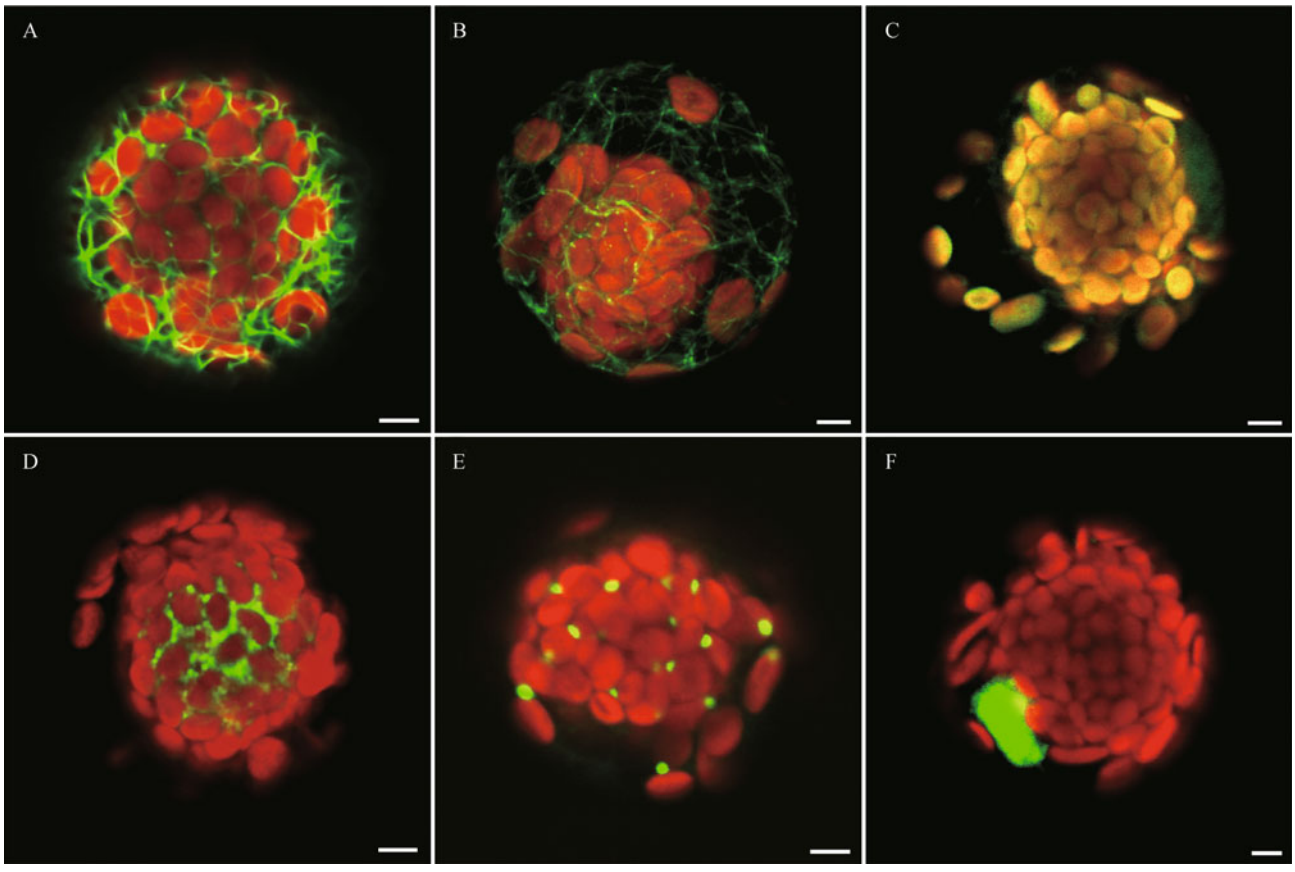

Figure 4 Transient expression of fluorescent fusion proteins in protoplasts of *Bienertia sinuspersici*. Transfected protoplasts were observed under a confocal laser scanning microscope. Merged images showing green fluorescent protein signals (green) and chlorophyll autofluorescence (red) in *Bienertia* protoplasts transfected with constructs of protein fusions to: A, talin, an actin binding protein; B, MAP4, a microtubule binding protein; C, Rubisco small-subunit; D, the transit peptide of NAD-malic enzyme; E, a peroxisomal targeting signal, or; F, a nuclear localization signal. Scale bars = 5 μm.

little is known about the compartmentation of single-cell C_4 machineries at the biochemical level. To this end, the precise distribution of organelle-specific enzymes must take place for proper functioning of the C_4 cycle. This requirement, however, implies that nuclear-encoded proteins must be specifically targeted to the correct subcellular compartment. Most chloroplast proteins are nuclear-encoded and translated in the cytosol as precursors with targeting sequences (Chen and Schnell, 1999). These preproteins are recognized and imported into the chloroplast by sophisticated proteinaceous apparatus located at the outer and inner membranes (the Tic/Toc complex; Translocon at the Inner/Outer envelope membrane of Chloroplasts) (Hirsch et al., 1994; Schnell et al., 1994). The core of the Toc complex consists of three proteins: Toc75, Toc159 and Toc34. Toc75 forms the protein translocation channel, whereas Toc159 and Toc34 are GTPase receptors for preprotein recognition and regulation of the import events. In *Arabidopsis*, four Toc159 homologs (i.e. atToc159, atToc132, atToc120 and atToc90) and two Toc34 homologs (i.e. atToc33 and atToc34) have been identified (Kubis et al., 2004). *In vivo* and biochemical data indicated that the substrate selectivity of the Toc complex is governed by different combinations of the Toc159 and Toc34 isoforms, such that atToc159 preferentially interact with atToc33 for the targeting and import of photosynthetic preproteins, whereas the Toc complex comprising of atToc120/132 and atToc34 is responsible for non-photosynthetic preproteins (Ivanova et al., 2004; Smith et al., 2004). Thus, the protein import function of the Toc complexes is essential for plastid biogenesis and differentiation.

In the single-cell C_4 system, the expression of C_4 genes is under the control of a single nucleus. Thus, during the different stages of leaf development the targeting of nuclear-encoded proteins destined for the dimorphic chloroplasts might involve a selective process to facilitate their proper development and intracellular compartmentation in order to achieve a functional C_4 pathway. As an initial step to understand the molecular mechanism regulating the differential accumulation of key photosynthetic enzymes in dimorphic chloroplasts, we have recently identified the core components of the Toc complex from the single-cell C_4 species (Lung and Chuong, 2012). In *B. sinuspersici*, the expression profiles of individual Toc159 isoforms appear to correlate with the development and differentiation of the

dimorphic chloroplasts. For example, Toc132 was more abundant in meristemic tissues where cells have undifferentiated proplastids, whereas high levels of Toc159 were observed in tissues containing photosynthetically active chloroplasts (Lung and Chuong, 2012). Moreover, immunoelectron microscopic studies using isoform-specific antisera revealed cytosolic and chloroplast envelope-associated localization of both Toc159 and Toc132, suggesting the possibility that these receptors can cycle between different compartments (Lung and Chuong, 2012). The immunolocalization results were further supported by fluorescent protein fusion studies in *B. sinuspersici* protoplasts as well as by immunoblot analyses of the Toc159 receptors in subcellular fractions (Lung and Chuong, 2012). Collectively, these results suggest that the Toc159 receptors are dynamic and their presence in various subcellular compartments may effectively facilitate their function in preprotein recognition and import. The notion that the Toc159 receptors are able to shuttle between the cytoplasm and chloroplast outer envelope has been previously proposed (Hiltbrunner et al., 2001; Ivanova et al. 2004; Smith, 2006). Further bioinformatics analyses of the Toc159 receptors of the single-cell C_4 system identified a novel transit peptide-like signal at the C terminus (Lung and Chuong, 2012). The function of this sorting signal to target proteins to the outer chloroplast envelope was confirmed using fluorescent fusion proteins in living protoplasts (Lung and Chuong, 2012). Despite the complexity of the subcellular anatomy and the differentiation of dual chloroplast types, our recent success in identifying the active core Toc components indicated that the general plastid protein import model also applies to the unique single-cell C_4 system. The presence of multiple isoforms of the Toc receptors supports the hypothesis that distinct Toc complexes present on each chloroplast type may be responsible for the differential import of proteins into dimorphic chloroplasts leading to their differentiation and establishment of the C_4 pathway in a single cell. With the advent of a novel technique for dimorphic chloroplast isolation (Lung et al., 2012), research is ongoing to test the differential targeting hypothesis using *in vitro* protein import assays.

The intracellular trafficking of macromolecules must involve the service of one or both components of the cytoskeleton in order to orchestrate their precise distribution. Since the operation of the single-cell C_4 systems requires strict partitioning of organelles and enzymes in distinct subcellular compartments, it is possible that proteins and/or mRNAs must be selectively targeted to the destined region of the cells to ensure the overall functional efficiency of the system. In addition to its role in maintaining the spatial separation of organelles and assisting their movement, it is possible that the cytoskeleton plays a role in the targeting of macromolecules to the proper subcellular compartments for specific cellular function. The involvement of the cytoskeleton in mRNA localization has been well documented in yeast, mammalian and plant cells (Bashirullah et al., 1998; Jansen, 2001; Crofts et al., 2005). The association of mRNAs with the cytoskeleton is important in concentrating the synthesis of proteins close to their site of function. Similarly, the cytoskeleton may participate in the intracellular protein trafficking systems to ensure that proteins are properly delivered to various subcellular compartments. In fact, a recent study reported the interaction between actin microfilaments and the Toc159 receptor (Jouhet and Gray, 2009). However, the role of the cytoskeleton in protein import into chloroplasts remains undetermined. Thus, future studies will focus on identifying and characterizing the molecular factors involved in the intracellular targeting of proteins in the single-cell C_4 systems, with an emphasis on the role of the cytoskeleton components in the process.

Conclusion and future directions

Bienertia and *S. aralocaspica* constitute an attractive biologic system to examine the molecular and biochemical modifications that underlie the shift from Kranz-type C_4 photosynthesis to single-cell C_4 type. These discoveries have led to a re-examination of the requirements for C_4 photosynthesis and raised questions about evolution of the Kranz-type C_4 system. In addition, they provide excellent examples of the ability of plant cells to establish and maintain biochemically complex compartments based on both partitioning of organelles and differential expression/targeting of gene products within the same cell. The single-cell C_4 systems represent the most complex examples of higher plant cells with respect to polar distribution of enzymes and organelles within a single cell. To further our understanding of these systems, research should be carried out to elucidate the nature of the cellular and molecular signals that trigger the onset of organelle movement leading to the establishment of two intracellular compartments within individual cells, since loss of this partitioning would probably prevent the C_4 cycle to function properly. Current and future studies will focus on providing new information about two key fundamental processes in plants: cellular organization leading to functional domains and photosynthetic carbon assimilation. Understanding the cellular and molecular mechanisms regulating the high-level of photosynthetic gene expression may potentially allow for the introduction of C_4-like characteristics into agriculturally important C_3 crops to increase productivity. This could be particularly important to confer a growth advantage to plants with C_4 or C_4-like features under current and changing climate conditions where increased water stress and reduced CO_2 solubility due to extreme temperatures and salinity.

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References

Akhani H, Barroca J, Koteeva N K, Voznesenskaya E V, Franceschi V, Edwards G, Ghaffari S, Ziegler H (2005). *Bienertia sinuspersici* (Chenopodiaceae): A new species from Southwest Asia and discovery of a third terrestrial C_4 plant without Kranz anatomy. Syst Bot, 30(2): 290–301

Akhani H, Chatrenoor T, Dehghani M, Khoshravesh R, Mahdavi P, Matinzadeh Z (2012) A new species of *Bienertia* (Chenopodiaceae) from Iranian salt deserts: A third species of the genus and discovery of a fourth terrestrial C_4 plant without Kranz anatomy. Plant Biosys,146(3): 550–559

Bashirullah A, Cooperstock R L, Lipshitz H D (1998). RNA localization in development. Annu Rev Biochem, 67(1): 335–394

Chen X, Schnell D J (1999). Protein import into chloroplasts. Trends Cell Biol, 9(6): 222–227

Chuong S D X, Franceschi V R, Edwards G E (2006). The cytoskeleton maintains organelle partitioning required for single-cell C_4 photosynthesis in Chenopodiaceae species. Plant Cell, 18(9): 2207–2223

Chuong S D X, Park N I, Freeman M C, Mullen R T, Muench D G (2005). The peroxisomal multifunctional protein interacts with cortical microtubules in plant cells. BMC Cell Biol, 6(1): 40

Collings D A, Lill A W, Himmelspach R, Wasteneys G O (2006). Drug sensitisation studies show actin microfilaments and microtubules interact during root elongation in *Arabidopsis thaliana*. New Phytol, 170: 275–290

Crofts A J, Washida H, Okita T W, Satoh M, Ogawa M, Kumamaru T, Satoh H (2005). The role of mRNA and protein sorting in seed storage protein synthesis, transport, and deposition. Biochem Cell Biol, 83(6): 728–737

Edwards G E, Franceschi V R, Voznesenskaya E V (2004). Single cell C_4 photosynthesis versus the dual-cell (Kranz) paradigm. Annu Rev Plant Physiol Plant Mol Biol, 55: 173–196

Edwards G E, Huber S C (1981). The C_4 pathway. In The biochemistry of plants, a comprehensive treatise. Vol.8. Photosynthesis, M.D. Hatch and N.K. Boardman, eds (N.Y., London, Toronto, Sydney, San Francisco: Acad. Press), pp. 237–281

Edwards G E, Voznesenskaya V E, Smith M, Koteyeva N K, Park Y I, Park J H, Kiirats O, Okita T W, Chuong S D X (2008). Breaking the paradigm in C_4 photosynthesis: Does it hold promise for C_4 rice? In: Sheehy J E, Mitchell P L, Hardy B. Charting New Pathways to C_4 Rice. Eds World Scientific Publishing Co. pp. 249–273

Edwards G E, Walker D A (1983). C_3, C_4: mechanisms, and cellular and environmental regulation, of photosynthesis. Oxford: Blackwell Scientific Publications

Freitag H, Stichler W (2000). A remarkable new leaf type with unusual photosynthetic tissue in a central Asiatic genus of Chenopodeaceae. Plant Biol, 2(2): 154–160

Hatch M D, Slack C R (1970). Photosynthetic CO_2-fixation pathways. Annu Rev Plant Physiol, 21: 141–163. In: Hatch M D, Osmond C B, Slatyer R O, eds (1971). Mechanism and function of C_4 photosynthesis. In: Photosynthesis and Photorespiration. New York: Wiley-Interscience, pp. 139–152

Hiltbrunner A, Bauer J, Vidi P A, Infanger S, Weibel P, Hohwy M, Kessler F (2001). Targeting of an abundant cytosolic form of the protein import receptor at Toc159 to the outer chloroplast membrane. J Cell Biol, 154(2): 309–316

Hirsch S, Muckel E, Heemeyer F, von Heijne G, Soll J (1994). A receptor component of the chloroplast protein translocation machinery. Science, 266(5193): 1989–1992

Ivanova Y, Smith M D, Chen K, Schnell D J (2004). Members of the Toc159 import receptor family represent distinct pathways for protein targeting to plastids. Mol Biol Cell, 15(7): 3379–3392

Jansen R P (2001). mRNA localization: message on the move. Nat Rev Mol Cell Biol, 2(4): 247–256

Jouhet J, Gray J C (2009). Interaction of actin and the chloroplast protein import apparatus. J Biol Chem, 284(28): 19132–19141

Kandasamy M K, Meagher R B (1999). Actin-organelle interaction: association with chloroplast in *Arabidopsis* leaf mesophyll cells. Cell Motil Cytoskeleton, 44(2): 110–118

Kubis S, Patel R, Combe J, Bédard J, Kovacheva S, Lilley K, Biehl A, Leister D, Ríos G, Koncz C, Jarvis P (2004). Functional specialization amongst the *Arabidopsis* Toc159 family of chloroplast protein import receptors. Plant Cell, 16(8): 2059–2077

Kwok E Y, Hanson M R (2003). Microfilaments and microtubules control the morphology and movement of non-green plastids and stromules in *Nicotiana tabacum*. Plant J, 35(1): 16–26

Lara M V, Offermann S, Smith M, Okita T W, Andreo C S, Edwards G E (2008). Leaf development in the single-cell C_4 system in *Bienertia sinuspersici*: expression of genes and peptide levels for C_4 metabolism in relation to chlorenchyma structure under different light conditions. Plant Physiol, 148(1): 593–610

Logan D C, Scott I, Tobin A K (2003). The genetic control of plant mitochondrial morphology and dynamics. Plant J, 36(4): 500–509

Lung S C, Chuong S D X (2012). A transit peptide-like sorting signal at the C terminus directs the *Bienertia sinuspersici* preprotein receptor Toc159 to the chloroplast outer membrane. Plant Cell, 24(4): 1560–1578

Lung S C, Yanagisawa M, Chuong S D X (2011). Protoplast isolation and transient gene expression in the single-cell C_4 species, *Bienertia sinuspersici*. Plant Cell Rep, 30(4): 473–484

Lung S C, Yanagisawa M, Chuong S D X (2012). Isolation of dimorphic chloroplasts from the single-cell C_4 species *Bienertia sinuspersici*. Plant Methods, 8(1): 8

Offermann S, Okita T W, Edwards G E (2011). Resolving the compartmentation and function of C_4 photosynthesis in the single-cell C_4 species *Bienertia sinuspersici*. Plant Physiol, 155(4): 1612–1628

Park J H, Knoblauch M, Okita T W, Edwards G E (2009). Structural changes in the vacuole and cytoskeleton are key to development of the two cytoplasmic domains supporting single-cell $C(_4)$ photosynthesis in *Bienertia sinuspersici*. Planta, 229(2): 369–382

Park J H, Okita T W, Edwards G E (2010). Expression profiling and proteomic analysis of isolated photosynthetic cells of the non-Kranz C_4 species *Bienertia sinuspersici*. Funct Plant Biol, 37(1): 1–13

Petrásek J, Schwarzerová K (2009). Actin and microtubule cytoskeleton interactions. Curr Opin Plant Biol, 12(6): 728–734

Sage R F (1999). Why C_4 photosynthesis? In C_4 Plant Biology. Physiological Ecology series, Sage R F and Monson R K, eds (San Diego: Academic Press), pp. 3–16.

Sage R F (2003). Atmospheric CO_2, environmental stress and the evolution of C_4 photosynthesis. In: Ehleringer J R, Cerling T E, Dearing D, eds. A History of Atmospheric CO_2 and its Effects on Plants, Animals and Ecosystems. Berlin: Springer-Verlag

Sampathkumar A, Lindeboom J J, Debolt S, Gutierrez R, Ehrhardt D W, Ketelaar T, Persson S (2011). Live cell imaging reveals structural associations between the actin and microtubule cytoskeleton in *Arabidopsis*. Plant Cell, 23(6): 2302–2313

Sato Y, Wada M, Kadota A (2001). Choice of tracks, microtubules and/or actin filaments for chloroplast photo-movement is differentially controlled by phytochrome and a blue light receptor. J Cell Sci, 114 (Pt 2): 269–279

Schnell D J, Kessler F, Blobel G (1994). Isolation of components of the chloroplast protein import machinery. Science, 266(5187): 1007–1012

Smith M D (2006). Protein import into chloroplasts: an ever-evolving story. Can J Bot, 84(4): 531–542

Smith M D, Rounds C M, Wang F, Chen K, Afitlhile M, Schnell D J (2004). atToc159 is a selective transit peptide receptor for the import of nucleus-encoded chloroplast proteins. J Cell Biol, 165(3): 323–334

Van Gestel K, Köhler R H, Verbelen J P (2002). Plant mitochondria move on F-actin, but their positioning in the cortical cytoplasm depends on both F-actin and microtubules. J Exp Bot, 53(369): 659–667

Voznesenskaya E V, Edwards G E, Kiirats O, Artyusheva E G, Franceschi V R (2003). Development of biochemical specialization and organelle partitioning in the single-cell C_4 system in leaves of *Borszczowia aralocaspica* (Chenopodiaceae). Am J Bot, 90(12): 1669–1680

Voznesenskaya E V, Franceschi V R, Kiirats O, Artyusheva E G, Freitag H, Edwards G E (2002). Proof of C_4 photosynthesis without Kranz anatomy in *Bienertia cycloptera* (Chenopodiaceae). Plant J, 31(5): 649–662

Voznesenskaya E V, Franceschi V R, Kiirats O, Freitag H, Edwards G E (2001). Kranz anatomy is not essential for terrestrial C_4 plant photosynthesis. Nature, 414(6863): 543–546

Voznesenskaya E V, Koteyeva N K, Chuong S D X, Akhani H, Edwards G E, Franceschi V R (2005). Differentiation of cellular and biochemical features of the single-cell C_4 syndrome during leaf development in *Bienertia cycloptera* (Chenopodiaceae). Am J Bot, 92(11): 1784–1795

Wada M, Kagawa T, Sato Y (2003). Chloroplast movement. Annu Rev Plant Biol, 54(1): 455–468

Wada M, Suetsugu N (2004). Plant organelle positioning. Curr Opin Plant Biol, 7(6): 626–631

Winter K, Smith J A C (1996). Crassulacean Acid Metabolism. New York: Spring