Endoreduplication is not involved in bundle-sheath formation in the C₄ species *Cleome gynandra*

Sylvain Aubry, Jana Knešová and Julian M. Hibberd*

Department of Plant Sciences, Downing Street, University of Cambridge, Cambridge CB2 3EA, UK

* To whom correspondence should be addressed. E-mail: jmh65@cam.ac.uk

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Abstract

There is currently significant interest in engineering the two-celled C₄ photosynthesis pathway into crops such as rice in order to increase yield. This will require alterations to the biochemistry of photosynthesis in both mesophyll (M) and bundle-sheath (BS) cells, but also alterations to leaf anatomy. For example, the BS of C₄ species is enlarged compared with that in C₃ species. Because cell and nucleus size are often correlated, this study investigated whether nuclear endoreduplication is associated with increased differentiation and expansion of BS cells. Nuclei in the BS of C₄ *Cleome gynandra* were tagged with green fluorescent protein. Confocal laser-scanning microscopy and flow cytometry of isolated nuclei were used to quantify size and DNA content in BS cells. The results showed a significant endoreduplication in BS cells of *C. gynandra* but not in additional C₄ lineages from both the monocotyledonous and dicotyledenous plants. Furthermore, in the C₃ species *Arabidopsis thaliana*, BS cells undergo endoreduplication. Due to this significant endoreduplication in the small BS cells of C₃ *A. thaliana*, it was concluded that endoreduplication of BS nuclei in C₄ plants is not linked to expansion and differentiation of BS cells, and therefore that alternative strategies to increase this compartment need to be sought in order to engineer C₄ traits into C₃ crops such as rice.

Key words: Bundle sheath, C₄ photosynthesis, *Cleome gynandra*, endoreduplication.

Introduction

From about 30 million years ago, plants using C₄ photosynthesis evolved, and they now populate more than 60 independent lineages of angiosperms (Christin *et al.*, 2013). The C₄ pathway reduces rates of photorespiration and therefore allows greater photosynthetic efficiency, primarily in open areas of the tropics and subtropics (Sage *et al.*, 2011). Although C₄ species only represent around 3% of angiosperms (Sage *et al.*, 2011), it is estimated that they contribute to about 30% of net terrestrial primary productivity (Osborne and Beerling, 2006).

The C₄ pathway involves the reactions of photosynthesis being divided between two compartments in the leaf, and this leads to CO₂ being concentrated in bundle-sheath (BS) chloroplasts in full C₄ plants that contain the primary carboxylase Ribulose Bisphosphate Carboxylase Oxygenase (RuBisCO).

In all cases, carbonic anhydrase and phosphoenolpyruvate carboxylase convert CO₂ into oxaloacetate in one compartment. The subsequent reduction or transamination of oxaloacetate to organic four-carbon acids such as malate and aspartate generate high concentrations of these metabolites, and this drives their diffusion into the second compartment. Here, one or more C₄ acid decarboxylase releases high concentrations of CO₂ around RuBisCO. In species that use the classic two-celled pathway, the high flux of metabolites between mesophyll (M) and BS cells is dependent on close contacts between these cell types, and typically this results in a stylized arrangement of each vein being surrounded by a ring of BS cells, which in turn is inside a ring of M cells, resulting in so-called Kranz anatomy (Hatch and Slack, 1966). The BS cells of C₄ plants also contain many chloroplasts to increase the...
The C₄ cycle is most often achieved by compartmenting photosynthesis between two cell types within the leaf, but it can occur within individual cells (Chuong et al., 2006; Lara et al., 2006). In species that use the two-celled C₄ pathway, there is considerable variation in the exact cells within the leaf that fulfill the initial phosphoenolpyruvate carboxylase-dependent carboxylation step and the subsequent decarboxylation and refixation by RuBisCO (Dengler et al., 1994). For example, there are at least 25 forms of Kranz anatomy (Edwards and Voznesenskaya, 2011) with four subtypes in the eudicots (Muhaidat et al., 2007) and the most common arrangement being the atriplicoid subtype (Muhaidat et al., 2007). The stylized pathway is often described as initial fixation in the M followed by decarboxylation in the BS, but in all cases, while in C₃ species M volume is larger than that of the BS, the converse is true in C₄ plants. This increase in volume of the BS in C₄ leaves can be caused by it containing either more and/or larger cells (Muhaidat et al., 2007; McKown and Dengler, 2009). As the increased productivity of C₄ plants has led to the proposal that characteristics of C₄ photosynthesis should be engineered into C₃ crops such as rice to increase yield (Matsuoka et al., 2001; Hibberd et al., 2008), we need to understand the genetic basis underlying the expansion, differentiation, and specialization of the BS in C₄ species.

In eukaryotes, there is often a positive correlation between cell size and nuclear DNA content (Sugimoto-Shirasu and Roberts, 2003; Lee et al., 2009). Polyploid nuclei can occur as a result of a process called endoreduplication where chromosomal DNA replication is not followed by mitotic cell division. Endoreduplication is therefore a result of the canonical cell cycle G₁-S-G₂-M missing a mitotic cell division, and, as a result, this leads to an increase in nuclear DNA content (De Veylder et al., 2007). In diploid organisms, endoreduplication can occur repeatedly during the lifetime of a cell, giving rise to multiple copies of the nuclear genome, ranging from 2C (the diploid state) up to 32–64C. In Arabidopsis thaliana, DNA content correlates with leaf age, and endoreduplication occurs once cells shift from proliferation to maturation (Beemster et al., 2005). In A. thaliana, cell division is arrested along a gradient from the tip to the base of the leaf during secondary morphogenesis (Donnelly et al., 1999). The physiological relevance of this gradual increase in nuclear DNA content is still a matter of discussion but can be induced during stress (Lee et al., 2009). It can also be found in tissues such as the maize endosperm (Schweizer et al., 1995) and hypocotyls of A. thaliana (Gendreau et al., 1998), and is common in trichomes (Kasili et al., 2010).

The correlation between nuclear ploidy state and cell size as well as organelle number has been reported in numerous plants (Kondorosi et al., 2000; Sugimoto-Shirasu and Roberts, 2003; De Veylder et al., 2011). For example, in floral apices of Datura stramonium, there is a direct correlation between ploidy, nucleus volume, and cell size, with larger cells having more DNA (Sugimoto-Shirasu and Roberts, 2003). In A. thaliana, ploidy levels of epidermal cells and trichomes show a positive correlation between DNA content and cell size (Melaragno et al., 1993; Hulskamp et al., 1999). While the direct link between ploidy and cell size is not always clear (Marks, 1997; John and Qi, 2008; Dismeyer et al., 2009), evidence that endoreduplication and cell enlargement are genetically linked is provided by analysis of Medicago sativa lines in which antisense repression of CCS52, which acts as a negative regulator of mitosis, led to both reduced ploidy and cell size (Cebolla et al., 1999). If a C₄ crop such as rice is to be engineered to use C₄ photosynthesis, the BS will need to be increased in size (Sage and Zhu, 2011). To inform the C₄ rice engineering effort, we therefore tested the hypothesis that expansion of the BS is associated with nuclear endoreduplication in this compartment. We demonstrate that the nuclei of BS cells of Cleome gynandra undergo endoreduplication, but that in other C₄ species from independent C₄ lineages within the angiosperms, this was not evident. Furthermore, we report that the endoreduplication in BS cells also occurred in C₃ A. thaliana. We therefore infer that, to engineer C₄ rice, alternate strategies will be needed to increase BS size.

Materials and methods

Cloning and production of transgenic lines

The vectors used were derived from the INTACT systems (Deal and Henikoff, 2010), which target green fluorescent protein (GFP) to the nuclear envelope. The FgGLDPAs (Wiludda et al., 2012) promoter was amplified as a 1200 bp fragment with XmaI and NheI restriction sites included in the primers XmaI-FgGLDPs-F 5′-CACCCCGGGAAGCTTTACTCCTCCTCAC-3′ and NheIFgGLDPs-R 5′-TTTGTCTAGCTAGTGAAGATGGGGGTCTA-3′. This allowed the fragment to be cloned into the GL2p:NTF vector (Deal and Henikoff, 2010) at the XmaI and NheI sites. The promoter-NTF region was then amplified by PCR and cloned into pENTR/D-TOPO with a CACC overhang on the 5′ primer to orientate the cloning. Clones containing the promoter and the NTF were sequenced and subsequently inserted into the binary vector pGW81 (Nakagawa et al., 2007) by Gateway LR recombination. Agrobacterium tumefaciens LBA4404 was transformed with this construct and selected on both kanamycin and streptomycin. LBA4404 was transformed with this construct and selected on both kanamycin and streptomycin. C. gynandra callus was transformed as described previously (Newell et al., 2010). Briefly, 10-d-old hypocotyls and cotyledons explants were sectioned and dipped in liquid MS medium supplemented with vitamins and 0.2 mM acetosyringone (pH 5.5) containing the resuspended A. tumefaciens culture. After 30 min, explants were transferred for 2 d to co-culture plates (MS medium at 1/10 normal concentration, 30 g L⁻¹ sucrose, 1 mg L⁻¹ of benzilamino-nopurine, 0.1 mg L⁻¹ of naphthyl acetic acid, 8 g L⁻¹ of agar, pH 5.5, covered with 1 ml MSO liquid), and finally placed on regeneration medium with antibiotics for 3 weeks. Explants were then grafted onto wild-type root stocks to allow seed production. Three independent T₁ transgenic lines were used in this study.

Plant material and microscopy

C. gynandra (C₃) and A. thaliana (C₄) were grown in soil under long-day conditions (16h light, 8h night) in a cabinet set at 150 µmol photons m⁻² s⁻¹ and a temperature of 23 °C during the day and 20 °C during the night. To assess endoreduplication, leaves were harvested from 5 to 30 d after planting and analysed by flow cytometry. For analysis of cell and nuclei size in M and BS cells, Atriplex rosea (Amaranthaceae), Flavera trinervia (Asteraceae), Zea mays (Poaceae), and Setaria viridis (Poaceae) were grown for 5 weeks,
Flow cytometry
DNA content was determined as described previously (Zhang et al., 2005; Dolezel et al., 2007) (see Supplementary Fig. S1, at JXB online). Briefly, 20 mg of fresh leaves was chopped with a razor blade for 30 s in 1 ml of 45 mM MgCl₂, 20 mM MOPS, 30 mM sodium citrate (pH 7) and 0.1% Triton X-100. The homogenate was filtered through a 40 μm nylon mesh and nuclei were stained with 2 μg ml⁻¹ of DAPI. Flow cytometry was performed on a Dako Cyan cytometer using 365 and 488 nm lasers. GFP fluorescence, excited by the 365 nm beam, was routed directly on the FL7 channel whereas nuclei in the M were scattered randomly within each. Areas of cells and nuclei were calculated using Photoshop CS6 from at least five independent biological replicates and statistically significant differences (P<0.05) were determined using a one-tailed t-test.

Results

BS nuclei are larger than those in mesophyll cells of C. gynandra

Confocal laser-scanning microscopy (CLSM) showed that, in mature leaves of C. gynandra, BS cells were 1.8 times larger than M cells (Fig. 1A, B). Propidium iodide staining of nuclei established that those in the BS were around three times larger than those in M cells (Fig. 1A, C). It was also apparent that nuclei in BS cells were often located between the centripetally arranged chloroplasts and the vacuole, whereas nuclei in the M were scattered randomly within each cell (Fig. 1A).

Endoreduplication in BS nuclei occurs in C₃ and C₄ species

To investigate whether the increase in BS size was associated with endoreduplication, we generated lines in which nuclei from BS cells contained GFP. Nuclei in BS cells were labelled using the BS-specific Flaveria trinervia glycine decarboxylase (FtGDLPA) promoter (Wiludda et al., 2012) to drive expression of a translational fusion between GFP and the WPP domain of the Ran GTPase activating protein (RanGAP1), which is targeted to the nuclear envelope (Deal and Henikoff, 2010, 2011) (Fig. 2A). CLSM confirmed that this construct led to specific expression in BS cells of C. gynandra (Fig. 2B, C). When nuclei from all cell types of the leaf were assessed, 80% were 2C, with only 18 and 2% being 4C and 8C, respectively (Fig. 2D, E; Supplementary Fig. S2 at JXB online). This was maintained from 5 to 30 d after germination as the leaves matured (Fig. 2E). In contrast, when fluorescently activated cell sorting was used to separate GFP-labelled BS nuclei from leaves, this established that the proportion of 2C and 8C nuclei declined and increased, respectively, as leaves matured (Fig. 2F, G).

The FtGDLPA promoter directs BS specificity in A. thaliana (Wiludda et al., 2012), and so to investigate whether the increased DNA content of BS nuclei is an ancestral characteristic, we transformed the same FtGDLPA::GFP::RanGAP1 construct into C₃ A. thaliana (Fig. 3A, B). When nuclei from all cells of A. thaliana leaves were separated and DNA content assessed, this showed
that there was a gradual reduction in 2C content as leaves matured and a consequent increase in 4C and 8C content (Fig. 3C, D). This trend was also evident in nuclei isolated from BS cells (Fig. 3E, F) and interestingly the increase in 8C nuclei in BS cells was very similar in C_{4} A. thaliana and C_{4} C. gynandra (Figs 2G and 3F).

To investigate the extent to which other C_{4} plants possess larger nuclei in BS compared with M cells, we analysed two additional C_{4} dicotyledons, Flaveria trinervia and Atriplex rosea, and also two monocotyledonous species, Zea mays (maize) and Setaria viridis. In contrast to C. gynandra, nuclei in BS and M cells were of a similar size in all four species.
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(Fig. 4 and Table 1). This was probably caused by endoreduplication in both cell types. Furthermore, the size of BS cells from *F. trinervia*, maize and *S. viridis* were not significantly larger than M cells (Table 1). Together, these data indicated that expansion of the BS compartment in these C4 species was due to a larger number, rather than larger size, of individual cells.

We also used flow cytometry to estimate the genome size of *C. gynandra*. Nuclei were extracted from *A. thaliana* and *C. gynandra* leaves and run separately and together on a flow cytometer (Fig. 5). Because the 2C genome of *A. thaliana* genome is known to be 135 Mb (http://www.arabidopsis.org), and 4C, 8C, and 16C content was also detectable, this allowed us to estimate that the genome of *C. gynandra* is approximately 956 Mb (Fig. 5).

**Discussion**

In *A. thaliana*, both M and BS cells undergo endoreduplication

By labelling and isolating nuclei from the BS of *A. thaliana*, we showed directly that endoreduplication can occur within...
BS cells of C₃ leaves. Furthermore, when nuclei were isolated from all leaf cell types, we still detected considerable amounts of endoreduplication. Because the BS only represents about 15% of all cells of the leaf (Kinsman and Pyke, 1998), we infer that endoreduplication had also occurred in M cells of *A. thaliana*. During leaf maturation in *A. thaliana*, there were marked alterations in the extent of endoreduplication, for example the number of 2C nuclei declined from 70 to 20% between 5 and 30 d after germination. Over the same time frame, the number of 4C and >8C nuclei increased from 30 and 15% to 55 and 35%, respectively. This large increase in ploidy as leaves of *A. thaliana* mature agrees with previous

**Table 1. Quantitative analysis of cell and nucleus area in* A. thaliana* and four C₄ species**

| Species      | C₄ subtype | Cell area (µm²) | Nucleus area (µm²) |
|--------------|------------|-----------------|--------------------|
|              |            | BS              | M                  | BS              | M               |
| *A. thaliana*| –          | 194.5±15.9**    | 319.4±17.2**       | 6.2±0.3         | 6.3±0.2         |
| *F. trinervia*| NADP-ME   | 130.5±7.5       | 127.4±8.5          | 28.4±1.4        | 32.1±1.5        |
| *A. rosea*   | NAD-ME     | 314.4±19.7*     | 244.0±12.4*        | 13.5±1          | 14.4±1.2        |
| *Z. mays*    | NADP-ME    | 246.0±46.6      | 356.2±23.2         | 31.6±3.4        | 27.9±1.1        |
| *S. viridis* | NADP-ME    | 213.4±14.3**    | 336.5±22.3**       | 9.3±0.7         | 6.3±0.3         |

*Fig. 4. BS and M nuclei from four species with independent C₄ origins. Representative transverse sections of leaves stained with propidium iodide and imaged with CLSM. Nuclei and cell walls are green, while chlorophyll fluorescence is red. Results are shown for *F. trinervia* (A), *A. rosea* (B), *Z. mays* (C), and *S. viridis* (D). Nuclei in M and BS cells are annotated with arrowheads and asterisks, respectively. Five biological replicates were assessed. Bars, 25 µm.*
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analysis of whole leaves and appears to correlate negatively with rates of cell division (Beemster et al., 2005). We did detect 4C nuclei or more in very young leaves, which could be due to either significant heterogeneity in the state of cell proliferation or to endoreduplication having occurred in specific cell types very early during leaf maturation (Donnelly et al., 1999).

Because we detected significant endoreduplication in the small BS cells of *A. thaliana* (Kinsman and Pyke, 1998), this implies that an increase in nuclear DNA content is not always linked to larger cells. This conclusion is consistent with previous work indicating that the positive correlation between ploidy levels and cell size is not ubiquitous. For example, increased ploidy in *A. thaliana* TRYPTYCHON (try) or KAKTUS (kak) mutants was not associated with increased trichome cell size (Marks, 1997), and in mutants of cyclin-dependent kinase A1, while cells were enlarged compared with wild type, nuclear DNA content remained stable (Dissmeyer et al., 2009). We also note that, although endoreduplication can be induced by stress (Lee et al., 2009), this was not required for it to be detected in the BS cells of *A. thaliana*.

Our data, combined with previous work, imply that endoreduplication in BS cells is an ancient and conserved characteristic of the angiosperms. As *A. thaliana* is phylogenetically closely related to *C. gynandra*, and their last common ancestor is thought to have diverged around 35 million years ago (Schranz and Mitchell-Ol's, 2006), endoreduplication within their BS cells may represent a characteristic present in the last common ancestor of these species. Endoreduplication in BS cells may in fact be even more ancient because it has also been reported in three species of monocotyledons from the Asparagales and the Liliiales (Olszewska et al., 1983). It is possible that endoreduplication within the BS evolved independently within these two lineages of the monocotyledons, and again in the Brassicales within the dicotyledons, but the most parsimonious explanation is that endoreduplication of BS nuclei is the ancestral condition within angiosperms.

While the role that the BS plays in C₃ plants is not certain (Leegood, 2002), photosynthesis in these cells impacts on whole plant growth and fitness (Janacek et al., 2009), and the cells themselves are implicated in responding to fluctuations in light intensity (Kangasjarvi et al., 2009) as well as in protection against cavitation (Christin et al., 2013; Griffiths et al., 2013). Whether endoreduplication in BS cells impacts on these key functions will be interesting to determine.

The role of endoreduplication in BS expansion of *C₄* plants

The increase in nuclear DNA content of both BS and M cells during leaf maturation in *A. thaliana* contrasts with the behaviour of nuclei from these cells in *C₄* *C. gynandra*. While it was clear that endoreduplication occurred within BS cells as leaves of *C. gynandra* matured, when nuclei from whole leaves were assessed, we did not detect an increase in DNA content. As the M of *C₄* *C. gynandra* represents a large proportion of the leaf, this indicates that little endoreduplication occurs in M cells of this species. We therefore conclude that, in *C₃* *A. thaliana*, endoreduplication occurs in both M and BS cells, but that in the *C₄* species *C. gynandra*, this behaviour becomes restricted to the BS.

The control of chloroplast number has previously been linked to both endoreduplication (Butterfass, 1989; Ho and Rayburn, 1991) and cell volume (Pyke and Leech, 1987). The fact that we detect significant endoreduplication in the small BS cells of *C₃* *A. thaliana* but also the large BS cells of *C₄* *C. gynandra* suggested that evolution of increased chloroplast number in BS cells of *C₄* species is not likely to be related to enreduplication in these cells but is co-ordinated with cell expansion. We also propose that endoreduplication is not required for expansion, differentiation, and specialization of

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**Fig. 5.** Genome size of *C. gynandra*. Flow cytometric analysis of DAPI-stained *A. thaliana* and *C. gynandra* wild-type leaves were performed either separately (A, B) or together (C). Log-scaled DAPI signals are plotted against the event counts. Based on the size of the *A. thaliana* genome (135 Mb), we inferred a genome size for *C. gynandra* of approximately 956 Mb. (D) Flow cytometric analysis of a *Tarenaya hassleriana* mature leaf, a closely related C₃ of *C. gynandra*.
the BS, a key characteristic of C₄ plants, but rather that it is a deeply rooted phenotype found within the angiosperms. Our reasoning for this is as follows. First, BS cells of C₃ A. thaliana undergo significant endoreduplication but are small compared with those of C₄ C. gynandra, and also compared with the M cells of C₃ A. thaliana. Secondly, because the large BS cells associated with Kranz anatomy in leaves of C. gynandra are present in very young leaves but endoreduplication occurs later during maturation, the two phenomena are unlikely to be linked. Thirdly, while individual BS cells of C₄ species such as C. gynandra (Fig. 1) (Marshall et al., 2007) and C₄ lineages within the Aizoaceae, Amaranthaceae, and Asteraceae (Muhaidat et al., 2007) are larger than C₃ relatives, it is also possible to increase the functional volume of the BS by increasing cell numbers rather than cell size. Because the BS surrounds the veins (Muhaidat et al., 2007), if the number of veins within a leaf increases, the consequence is a larger BS compartment. If the BS as a whole is enlarged because of additional cell division, the individual cells within it could remain the same or actually be smaller. In fact, in F. trinervia, A. rosea, Z. mays, and S. viridis, we did not detect large differences in the size of M and BS nuclei, and in maize, F. trinervia, and S. viridis, individual BS cell area was not larger than that of M cells. This is consistent with previous reports that have documented similar M and BS cell sizes in maize (Pengelly et al., 2011), sorghum, Urochloa panicoides (von Caemmerer et al., 2007) and Flaveria bidentis (Pengelly et al., 2010). Taken together, the data indicate that alterations to the M:BS ratio often occur independently of alterations to individual cell size and that there is not a simple link between endoreduplication and expansion and specialization of BS cells in C. gynandra.

**Supplementary data**

Supplementary data are available at JXB online.

Supplementary Fig. S1. Flow chart of sample preparation and biparametric cytometric analysis to determine BS ploidy levels in A. thaliana and C. gynandra.

Supplementary Fig. S2. Image of Cleome gynandra leaves used for flow cytometry analysis. Numbers represent days after germination.

Supplementary Fig. S3. Representative flow cytometry profile of 18 000 nuclei of Arabidopsis leaves 10 days after germination sorted according to DAPI and GFP fluorescence.

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