INTRODUCTION

The functional development of a variety of differentiated cell types is an essential component of leaf development. The dicotyledonous leaf is a relatively simple structure composed of only a few differentiated cell types, which arise from leaf primordial cells derived from the shoot apical meristem. Although leaf growth and development have been well characterised in a number of dicotyledonous species, very few studies in leaf development have utilised molecular genetic tools. The exploitation of Arabidopsis thaliana should allow for the genetic dissection of leaf development. The Arabidopsis leaf has a simple shape and a relatively simple cellular structure (Pyke et al., 1991) and maintains a basal plate meristem as cells in distal parts of the leaf expand. Although specific cell types present in leaves, such as trichomes and guard cells, have been subjected to mutational analysis (Larkin et al., 1997), the major chloroplast-containing cells within the leaf have not been studied in detail. In this study we have focused on characterising the chloroplast-containing cells which surround the vascular bundle within the leaf mesophyll tissue. These cells have been called by various names in different species but in this paper we have followed Esau (Esau, 1953), who used the term bundle sheath cell.

Bundle sheath cell development has been well characterised in grasses with C_4 metabolism (Dengler et al., 1985; Eastman et al., 1988; Dengler et al., 1994), particularly maize (Langdale et al., 1989; Nelson and Langdale, 1989), mainly because bundle sheath differentiation results in cell-specific gene expression and plastid development, leading to the compartmentation of different photosynthetic enzymes in the bundle sheath and mesophyll cells (Langdale et al., 1988a,b; Bansal and Bogorad, 1993). The maize mutants, bsd1 and bsd2, which show cell-specific mutant phenotypes, with abnormal bundle sheath cells and normal mesophyll cells, have been characterised and may provide an insight into cell-specific gene regulation in the C_4 system (Langdale and Kidner, 1994; Roth et al., 1996). Cell-specific gene expression and ultrastructural changes are also a feature of improved photosynthetic carbon reduction efficiency in C_3-C_4 intermediate species (Morgan et al., 1993; Devi et al., 1995).

In comparison, the development and function of bundle sheath cells of C_3 plants have received little attention. The few studies on development of bundle sheath cells in C_3 dicotyledonous species have been largely descriptive (Dengler and MacKay, 1975; Dengler et al., 1975), although Armacost (Armacost, 1945), using the term ‘border parenchyma’, proposed a number of functions for bundle sheath cells including transport of water and photosynthetic intermediate storage and a mechanical support function. It is now generally assumed that bundle sheath cells function in phloem loading and unloading, although the precise nature of this role is unclear (van Bel, 1992; van Bel, 1993). Bundle sheath cells have been largely overlooked as a cell type in Arabidopsis.
leaves, and a developmental genetic approach to dissecting aspects of their development and function has not previously been undertaken.

The characterisation of Arabidopsis mutants in which there is a visible differential development between mesophyll cells and the cells surrounding the vascular tissue is likely to be useful in determining how the differentiation processes of bundle sheath cells and their chloroplasts occur in relation to the development of the vascular strand, and to identify genes whose expression is bundle sheath- or mesophyll cell-specific. Although a large collection of reticulate leaf mutants is available in Arabidopsis Stock Centres, in which the vascular pattern can be distinguished on the lamina due to colour difference, i.e. green vasculature on a pale lamina, only two reticulate mutants have been named and mapped; reticulata (Redei and Hirono, 1964), which has dark green venation on a green lamina, and cue1, which has green venation on a pale lamina (Li et al., 1995). In this study, we have examined collections of previously uncharacterised reticulate leaf mutants in order to analyse the genetic control of the development of bundle sheath cells and their chloroplasts in Arabidopsis leaves.

MATERIALS AND METHODS

Plant material

Seeds of Arabidopsis thaliana (L.) ecotype Landsberg erecta were sown as previously reported (Pyke et al., 1991) and grown under natural light supplemented to provide a 16:8 hours light:dark photoperiod at 20°C. Analysis of cellular leaf development was confined to the first pair of true leaves, unless otherwise noted, since their general cellular development has been well characterised previously (Pyke et al., 1991). Seeds of mutants with reticulate leaf phenotypes were obtained from the Nottingham Stock Centre (UK) and the Arabidopsis Biological Resource Center, Columbus, Ohio (USA) and grown as described for Landsberg erecta.

Anatomical analysis

Leaves were fixed for 2 hours in 0.1 M piperazine-N,N′-bis(2-ethanesulfonic acid) (PIPES) buffer containing 3% (v/v) glutaraldehyde and 4% (v/v) paraformaldehyde (pH 7.6). After postfixation in 1% (v/v) aqueous osmium tetroxide, tissue was dehydrated and embedded in Spurr resin. Sections of 1 μm thickness were cut on a Cambridge-Huxley microtome using glass knives and stained for 30 seconds at 60°C with toluidine blue. Sections were viewed under a Nikon Optiphot microscope (Nikon, Tokyo) and quantitative analysis of leaf and cellular anatomical features was carried out using a Lucia image analysis system (Nikon, Tokyo) linked to the microscope by a TK-128OE JVC CCD camera. For ultrastructural analysis, ultra-thin sections were cut and stained with uranyl acetate and Reynolds lead stain and examined using a Zeiss EM109 transmission electron microscope.

Analysis of vascular pattern

In order to determine how the vascular system extends during leaf expansion, first leaves at various developmental stages were cleared in 95% ethanol at 4°C for 24 hours and photographed using dark-field illumination on a Leica Labolux-S microscope (Leica, Heerbrugg, Switzerland). Colour photographic slides were scanned using a Nikon SuperCoolscan film scanner (Nikon, Tokyo) and images were imported into the Lucia image analysis system for measurement of leaf area and vascular length.

Leaf cell separation

In order to view isolated bundle sheath and mesophyll cells, Arabidopsis first leaves were vacuum-infiltrated with a digestion buffer consisting of 0.1 M sodium phosphate buffer (pH 7.2) containing 0.3 M mannitol and 0.3% (w/v) Driselase (Sigma) and incubated at 25°C for 1 hour. Cells were then fixed and separated as described previously (Pyke and Leech, 1992). Suspensions of separated cells were tapped out from pieces of processed leaf tissue mounted in 0.1 M disodium ethylenediaminetetraacetic acid (Na₂EDTA) (pH 9) on microscope slides. Using Nomarski optics, numbers of chloroplasts per cell were counted and areas of individual cells and plastids were measured using the Lucia image analysis system.

Confocal microscopy

Images of chlorophyll fluorescence in intact, unfixed leaves mounted in Vectashield (Vector Laboratories, Peterborough) were obtained using a Leica TCS 4D confocal laser scanning microscope. Red chlorophyll autofluorescence was visualised in chloroplasts using the tetramethylrhodamine B isothiocyanate (TRITC) excitation channel according to the manufacturer’s software.

RESULTS

Vascular pattern development in Arabidopsis leaves

Since bundle sheath cells are vascular-associated, we first analysed the timing of vascular development in relation to leaf expansion. From observations of vascular pattern development, we have defined four levels of vascular hierarchy in the mature first leaf. The 1° vascular strand present in developing primordia derives from the vasculature of the hypocotyl (Fig. 1A). It extends into the primordium and bifurcates at the distal end forming two peripheral strands that rejoin the midrib midway along its length (Fig. 1B). This junction is often asymmetrical and forms a recognisable landmark in the vascular pattern (Fig. 1F). 2° vascular strands cross-link the 1° system and are connected to the main strand toward the leaf base (Fig. 1C). The 3° and 4° strands are formed at around 5% of final expanded size (Fig. 1D-F). By maturity the vascular pattern consists of approximately 25% of each level of the hierarchy (Fig. 2). Both vascular length and the number of vascular cells per unit leaf area decline during early development (Fig. 2C), and from 50% of final leaf size, the level of vascular density is maintained into full expansion (Fig. 2C). This increase in production in vasculature is contributed to by all levels of vascular hierarchy since the proportion of each level at 50% of full expansion is similar to that at maturity (Fig. 2C). The basic pattern of vascular development appears similar in later leaves, although there are some differences, particularly related to the termination of vascular strands at the leaf margin and the degree of leaf serration (data not shown).

Bundle sheath cells of Arabidopsis leaves

Bundle sheath cells form a sheath around the vascular strands, consisting principally of a single layer of cells (Fig. 3), the basic morphology of which is similar throughout the entire vascular system of the leaf. The typical bundle sheath consists of six cells, although this can vary from four to twelve cells depending on the thickness of the vascular strand and the order of the vein (Fig. 3A,B). Direct contact of the vascular strand with airspace is very rare, although direct contact of the bundle sheath cells with airspace is commonly observed (Fig. 3A,B). Bundle sheath cells are generally elongate in the direction of...
the vascular axis, with either perpendicular or oblique end walls (Fig. 3C,D), which occasionally leads to an overlap of cells and a double cell layer for a short distance. Whilst most bundle sheath cells have parallel side walls, some are more irregular with protrusions into the mesophyll tissue (Fig. 3C), and, at vascular junctions, individual bundle sheath cells follow the contour of the vascular strand, resulting in curved bundle sheath cells (Fig. 3E,F).

Bundle sheath cells are tightly associated with the vascular strands and in cell preparations using the Na$_2$EDTA cell separation method (Pyke and Leech, 1992) bundle sheath cells are rarely seen in isolation. A factor which probably contributes to the tight association between bundle sheath cells and vascular strands is the proportion of bundle sheath cell surface in contact with neighbouring cells (85% of bundle sheath cell wall is in contact with its neighbour, compared with 47% for mesophyll cells). Treatment of leaves with Driselase, a crude cocktail of cell wall digesting enzymes, improves the release of bundle sheath cells from their vascular associations, and large groups of separated bundle sheath cells can be observed, either in association with vascular strands (Fig. 4A) or as isolated groups of cells (Fig. 4B). Bundle sheath cells are smaller than mesophyll cells (Table 1) and identifiable by their elongate morphology and end on cell-cell contact (Fig. 4C). From measurements of bundle sheath cell size and vascular length, we calculate that the Arabidopsis first leaf contains about 7000 bundle sheath cells. The total number of mesophyll plus bundle sheath cells has previously been estimated at 48000 (Pyke et al., 1991). Bundle sheath cells therefore constitute a significant 15% of chloroplast-containing cells within the leaf. Bundle sheath cells also contribute significantly to the dimensions of the vascular bundle. We calculate that the bundle sheath confers a greater than 4-fold increase to the cross-sectional area of the vascular bundle compared to naked strand, with a doubling of its perimeter.

Longitudinal sections of leaf primordia show the presence of both periclinal and anticlinal planes of cell division in cells adjacent to a developing vascular strand (Fig. 5), suggesting that bundle sheath cells differentiate in a position-specific manner, rather than from a distinct cell lineage. Even at this early stage, elongation of the developing bundle sheath cell
adjacent to the vascular strand is apparent (Fig. 5), suggesting that the two cell types expand in concert.

**Bundle sheath cell chloroplasts**

Bundle sheath cell chloroplasts show several features which distinguish them from their counterparts in mesophyll cells. Although the internal ultrastructure of bundle sheath cell chloroplasts is similar to mesophyll cell chloroplasts, and thylakoid structure and granal stacking appear normal (data not shown), bundle sheath cell chloroplasts are smaller and occur at a lower density in the cell (Table 1). Consequently, the total chloroplast plan area per cell (i.e. the product of mean chloroplast size and chloroplast number per cell) is greatly reduced in bundle sheath cells relative to mesophyll cells, as shown by a reduced cell index (Table 1). Bundle sheath chloroplasts are often positioned on the cell wall distal to the vascular strand (Fig. 3) and are largely absent from the end walls and the wall directly connected to the vascular strand.

**dov1, a reticulate leaf mutant of Arabidopsis**

In order to identify mutants in which chloroplast development may be differentially perturbed in either bundle sheath or mesophyll cells, we screened approximately 200 mutants from Arabidopsis Stock Centre collections which were listed as possessing a reticulate leaf phenotype, e.g. green vascular pattern on a paler lamina. From these we have identified several mutants which have been named dov (differential development of vascular associated cells). We have identified a mutant, dov1 (Nottingham Stock Centre accession N557), the leaves of which show a clear reticulate leaf phenotype of a green vascular pattern on a pale lamina, which is apparent at very early stages of leaf development (Fig. 6). The dov1 mutant segregates as a single recessive nuclear mutation and is not allelic to two other characterised reticulate Arabidopsis mutants, reticulata and cue1. The dov1 phenotype is leaf specific, since the cotyledons

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**Fig. 3.** Sections of vascular bundles from fully expanded first leaves of Arabidopsis. (A,B) Transverse sections showing sheaths composed of (A) 11 and (B) 7 bundle sheath cells. (C,D) Longitudinal sections of vascular bundles showing the files of elongated bundle sheath cells immediately adjacent to the vascular tissue. The irregular-shaped bundle sheath cells in C are indicated. (E,F) Longitudinal sections of vascular junctions showing the curved or irregular morphology of individual bundle sheath cells as they follow the contour of the vascular junction. Bars, 50 μm.

**Fig. 4.** Bundle sheath and mesophyll cells isolated from fully expanded first leaves of Arabidopsis after Driselase treatment, followed by fixation and Na₂EDTA separation (see Materials and Methods). (A) An isolated vascular bundle showing files of bundle sheath cells still tightly associated with the vascular strand. (B) An isolated group of intact bundle sheath cells. (C) Isolated bundle sheath and mesophyll cells. Two bundle sheath cells joined by an oblique end wall and a bundle sheath/mesophyll cell junction (arrows). Bar, 50 μm.
and other green plant parts, such as sepals and petioles, are normal. The vascular pattern in dov1 leaves is similar to wild type and shows a similar vascular density in equivalent leaves (data not shown). Whole plants and leaves of the dov1 mutant are smaller than wild type (Fig. 6A,B), in common with most pale reticulate lines, but have normal flowering and fertility. There is tight resolution of the green reticulate phenotype to the underlying vascular pattern (Fig. 6C,D). Characterisation of fixed, separated cells from dov1 leaves reveals that bundle sheath cells and their chloroplasts always appear normal, whereas the chloroplasts in both palisade and spongy mesophyll cells are mostly pale and appear abnormal (Fig. 7A,B). Occasionally, a few mesophyll cells are observed to have apparently normal green chloroplasts, often mixed with abnormal pale chloroplasts in the same cell. Confocal images of chlorophyll fluorescence in dov1 leaves (Fig. 7C) reveal normal bundle sheath cells, but very abnormal mesophyll tissue with severely disrupted mesophyll chloroplasts in which chlorophyll autofluorescence is severely reduced. Longitudinal sections through dov1 leaves (Fig. 7D) confirm that bundle sheath cells and their chloroplasts appear normal whereas mesophyll cells, whilst having normal cell morphology and size, have dramatically perturbed chloroplasts. Chloroplast number per cell in dov1 mesophyll cells is reduced by 57% compared to wild type (Table 1). Chloroplast size is similar but cell index in dov1 mesophyll cells is only 30% that of wild-type mesophyll cells (Table 1). In contrast, measurements of dov1 bundle sheath cells and their chloroplasts do not show any major differences compared to wild type (Table 1). Even though chloroplast size in dov1 isolated mesophyll cells is similar to wild type, it is obvious that these chloroplasts are very pale and abnormal. This was confirmed by ultrastructural analysis of dov1 chloroplasts (Fig. 8) which reveals dramatically altered internal structure of mesophyll cell chloroplasts whereas dov1 bundle sheath cell chloroplasts are normal. dov1 mesophyll cell chloroplasts are largely agranal, contain many plastoglobuli and show extensive vacuolation of the chloroplast envelope (Fig. 8).

### DISCUSSION

We have clearly defined bundle sheath cells in Arabidopsis as a specific differentiated leaf cell type with a recognisable cellular morphology and position within the leaf, which previous studies on Arabidopsis leaves have largely failed to distinguish (Pyke et al., 1991). It is clear that the bundle sheath is fundamental to leaf function, that the recruitment of cells to form the bundle sheath is closely associated with vascular development and that the adjacent vascular tissue causes cell elongation parallel to the vein at the earliest stage of recruitment into the bundle sheath cell differentiation pathway. It is possible that the role of auxin in vascular differentiation is a factor in the orientation of bundle sheath cell expansion (Sachs, 1991) but there is no evidence from our study that bundle sheath cells develop in a lineage-dependent manner, and it is likely that vascular derived factors play a primary role in bundle sheath cell differentiation, as has been suggested in maize (Langdale et al., 1995).

Whilst it may be convenient to consider bundle sheath cells as being intermediate in form and function between vascular and mesophyll tissue, this may well be an oversimplification of their status in the C3 leaf. Bundle sheath cells share the polarity of their vascular neighbours and function in the transport of water and photoassimilate. Like their mesophyll neighbours, however, they are chlorophyllous and highly vacuolate and constitute a significant proportion of their photosynthetic capacity. However, the existence of bundle sheath-like cells in other species in the absence of adjacent vascular tissue provides further evidence for the distinctive nature of this cell type. The paraveinal mesophyll in soybean leaves, which lies between the palisade and spongy mesophyll, provides cellular continuity with the bundle sheath, and is

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**Table 1. Mean values for cell size, chloroplast number, chloroplast size and cell index in mesophyll and bundle sheath cells from first leaves of wild-type and the dov1 mutant of Arabidopsis**

<table>
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<th>Wild type</th>
<th>dov1</th>
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<tr>
<td></td>
<td>Mesophyll cells</td>
<td>Bundle sheath cells</td>
</tr>
<tr>
<td>Mesophyll cell plan area in ( \mu m^2 ) (n=40)</td>
<td>1806 (119)</td>
<td>2330 (165)</td>
</tr>
<tr>
<td>Chloroplasts per mesophyll cell (n=40)</td>
<td>76 (5)</td>
<td>32 (3)</td>
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<tr>
<td>Chloroplast plan area in ( \mu m^2 ) (n&gt;180)</td>
<td>39.6 (0.4)</td>
<td>39.5 (0.9)</td>
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<tr>
<td>Cell index</td>
<td>1.7</td>
<td>0.5</td>
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<tr>
<td>Bundle sheath cell plan area in ( \mu m^2 ) (n=40)</td>
<td>571 (27)</td>
<td>752 (46)</td>
</tr>
<tr>
<td>Chloroplasts per bundle sheath cell</td>
<td>22 (0.9)</td>
<td>25 (1.5)</td>
</tr>
<tr>
<td>Bundle sheath chloroplast plan area in ( \mu m^2 ) (n&gt;160)</td>
<td>24.6 (0.4)</td>
<td>20.7 (0.4)</td>
</tr>
<tr>
<td>Cell index</td>
<td>0.9</td>
<td>0.7</td>
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Cell index is calculated as (chloroplast number per cell × mean chloroplast plan area)/cell plan area. Standard errors are given in parentheses.
thought to function in the transfer and compartmentation of assimilates prior to export (Franceschi and Giaquinta, 1983). Equally, the ‘distinctive cells’ in some C₄ species of certain genera, e.g. Arundinella and Arthraxon (Ueno, 1995; Dengler et al., 1996) bear strong similarity to bundle sheath cells, but arise in distinct cell files which have no association with vascular tissue (Dengler et al., 1996). It is noteworthy that vein spacing in Arundinella hirta is unusually wide for a C₄ grass, and it is suggested that files of distinctive cells are degenerate veins (Dengler et al., 1996, 1997). Consequently, in some systems some aspects of bundle sheath cell differentiation could be semi-autonomous and not vascular dependent. Thus, it is possible that in Arabidopsis bundle sheath cells exist as a distinct, specified cell type whose differentiation may not arise solely from positional information.

The position and morphology of bundle sheath cells within the leaf suggests that their role in leaf physiology is probably multifunctional. Bundle sheath cells appear photosynthetically competent since they contain a significant chloroplast population with normal internal morphology, and 20% of the bundle sheath is exposed to intercellular airspace. Consequently these cells could contribute significantly to overall leaf photosynthesis. The high proportion of bundle sheath cell surface area in contact with adjacent cells suggests a central role for these cells in the transport of water and solutes into the mesophyll (Esau, 1953). The presence of irregularly shaped bundle sheath cells with lobes extending out into airspaces has also been observed in beech leaves (Dengler and MacKay, 1975). This characteristic may serve to increase the surface area of the vascular bundle and may enhance humidification of intercellular spaces. The position of bundle sheath cells makes them strong candidates for a role in the transfer of photoassimilate from mesophyll to phloem in source leaves. Phloem loading in Arabidopsis is probably apoplastic (Sjölund, 1997), but phloem loading is denoted apoplastic if plasmodesmata are virtually absent at some point in the pathway, irrespective of the site of symplastic discontinuity (van Bel, 1993). Hence, the precise role of Arabidopsis bundle sheath cells in phloem loading remains to be elucidated.

The bundle sheath may confer mechanical strength within the leaf. The tight attachment of the bundle sheath to the vascular strand appears to be greater than would be expected on the basis of the proportion of cell-cell contact. Furthermore, the arrangement of the vascular bundle as a cylinder composed of a ring of smaller cylinders enclosing the vascular strand may increase mechanical strength. The mechanical behaviour of a tissue is governed by the characteristics of both the cell wall and the cell contents, and by the composite of the two, i.e. the
relative density of the cell (Niklas, 1989). By having its vascular strand enclosed within a cylinder of turgid cells, the leaf is better able to withstand compressive and tensile forces and thus prevent buckling which could disrupt vascular continuity.

Efficient nutrient recycling from a senescent leaf requires that senescence must proceed in a broadly basipetal direction and that the vascular bundle must stay functional for longer than mesophyll cells. The pattern of senescence in Arabidopsis leaves supports this (Bleecker and Patterson, 1997). It is possible that senescence-regulated genes are expressed in a cell-specific manner, although molecular genetic characterisation of senescence-related genes has not addressed cell specificity to date.

Whilst there is no evidence that the metabolic function of Arabidopsis bundle sheath cells and their chloroplasts differs significantly from mesophyll cells, such a difference cannot be ruled out unequivocally. Clearly, the cell-specific compartmentation of photosynthetic enzymes of C4 plants does not hold in C3 plants. However, C4 photosynthesis arose as an adaptation of the C3 pathway and the transition from C3 to C4 photosynthesis arose independently on several occasions during the course of evolution (Ku et al., 1996), suggesting an inherent propensity for such distinctive differentiation between the two cell types. Our characterisation of the dov1 mutant provides evidence that differences in development between bundle sheath and mesophyll cell chloroplasts do exist in C3 leaves such as Arabidopsis, but are likely to be more subtle than those well characterised differences in C4 systems. Although the genetic basis for the dov1 mutation is unclear, it is apparent that the mutation affects mesophyll cell chloroplasts from an early stage in development since the extent of their division is perturbed, in addition to which their internal structure is highly abnormal. Furthermore, we have observed that perturbations in mesophyll chloroplast development can be influenced by environmental conditions in this mutant (data not shown). Whilst we have shown that, in wild-type plants, chloroplasts in bundle sheath cells are different from those in mesophyll cells in the extent of chloroplast division and expansion, other studies have provided evidence for cell-specific differences in gene transcription. Transformation of C3 rice with reporter genes driven by C4 mesophyll cell-specific promoters results in light-regulated, mesophyll cell-specific expression in the transformants. Thus, C3 plants have a regulatory system which is able to direct cell-specific and light regulated expression of ‘C4-type’ genes (Matsuoka et al., 1993, 1994).

The preferred positioning of bundle sheath cell chloroplasts on the cell surface distal to the vascular strand may reflect chloroplast function. However, one explanation for this distribution comes from the analysis of chloroplast distribution in mesophyll cells of Arabidopsis, in which those portions of the cell which are in direct contact with adjacent cells have few or no chloroplasts, and chloroplasts are preferentially positioned on the cell surface adjacent to airspace (Pyke, unpublished data). The major area of bundle sheath cell wall exposed to air space is that most distal to the vein. We suggest that this may be the underlying cause of the peripheral chloroplast localisation in the bundle sheath.

Our detailed characterisation of the bundle sheath cell type in Arabidopsis has shown the distinctive nature of this cell type. It has also revealed differential chloroplast development between bundle sheath cells and mesophyll cells. This raises the potential for analysing differential cell-specific gene expression between bundle sheath and mesophyll cells, and should enable us to gain a greater understanding of the function of this cell type in Arabidopsis leaves.

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