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**SPECIAL ISSUE REVIEW PAPER**

# Roles of the bundle sheath cells in leaves of $C_3$ plants

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## Abstract

**This review considers aspects of the structure and functions of the parenchymatous bundle sheath that surrounds the veins in the leaves of many $C_3$ plants. It includes a discussion of bundle sheath structure and its related structures (bundle sheath extensions and the paraveinal mesophyll), its relationship to the mestome sheath in some grasses, and its chloroplast content. Its metabolic roles in photosynthesis, carbohydrate synthesis and storage, the import and export of nitrogen and sulphur, and the metabolism of reactive oxygen species are discussed and are compared with the role of the bundle sheath in leaves of $C_4$ plants. Its role as an interface between the vasculature and the mesophyll is considered in relation to the movement of water and assimilates during leaf development, export of photosynthates, and senescence.**

Key words: Bundle sheath, $C_3$ plants, $C_4$ photosynthesis, compartmentation, leaf metabolism.

## Introduction

The bundle sheath in a leaf is a layer of compactly arranged parenchyma surrounding the vasculature (Esau, 1965) and is a conduit between the vasculature and the mesophyll cells. Bundle sheath cells constitute ~15% of chloroplast-containing cells in an *Arabidopsis* leaf (Kinsman and Pyke, 1998), and they conduct fluxes of compounds both into the leaf, particularly during leaf development, and out of the leaf, during export of photosynthates and during senescence. The bundle sheath also conducts the flow of water from the xylem to the mesophyll cells and then to the intercellular spaces. The bundle sheath cells are the only cells outside the vasculature itself (xylem, phloem, and some of their associated parenchyma cells) through which these substances must pass. The bundle sheath of $C_3$ plants maintains hydraulic integrity to prevent air entering the xylem, and may also store water to buffer transpirational surges that can be common in arid tropical climates (Sage, 2001).

Although studies of the activities of single cells or groups of cells in plants are, to a large degree, still in their infancy, there is evidence for intercellular compartmentation of activities between the different types of cells of the vasculature. Clearly the xylem and phloem have distinct functions, and the phloem companion cells are themselves considered highly metabolically active (van Bel and Knoblauch, 2000). However, in addition, there is evidence for separate metabolic and transport activities that are partitioned between the xylem parenchyma, phloem parenchyma, and the bundle sheath(s). For example, it is clear that phloem parenchyma is involved in the pathway of solutes to the phloem, and that it plays a role in carbohydrate metabolism (Nolte and Koch, 1993), and that xylem parenchyma is involved in the pathway of water and solutes from the xylem, for example, amino acid uptake from the xylem (Okumoto *et al*., 2002). It is also apparent that these cell types play more unexpected roles, for example, in secondary product synthesis (Burlat *et al*., 2004) and wound signalling (Hilaire *et al*., 2001; Narváez-Vásquez and Ryan, 2004). In $C_4$ plants, the bundle sheath cells have been recruited to a very specific role in photosynthetic $CO_2$ fixation, in which they form a specialized compartment in which $CO_2$ can be concentrated around Rubisco, thus suppressing photorespiration (von Caemmerer and Furbank, 2003), but this is in addition to other possible functions.

The aim of this paper is principally to review the known metabolic and transport activities of the bundle sheath cells of $C_3$ leaves. Are the bundle sheath cells just a conduit for solutes, or are they actively involved in their metabolism? Although it is tempting to infer a role for the bundle sheath in the metabolism and exchange of solutes

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between the vasculature and the mesophyll, it must be remembered that we are equally ignorant about the degree of specialization of mesophyll cells at various distances from the bundle sheath and the vasculature (Walter *et al*., 2004). For comparison, attention is drawn to the division of labour between the bundle sheath and mesophyll in various studies made of $C_4$ plants (the study of which is made much easier because of the robustness of the bundle sheath, which allows investigation of intercellular compartmentation). The allocation of various non-photosynthetic activities to the $C_4$ bundle sheath may have nothing to do with $C_4$ photosynthesis *per se*, but may simply reflect activities that already occurred in the bundle sheath of these plants before $C_4$ photosynthesis evolved. Thus it may be instructive to investigate global patterns of gene expression (Wyrich *et al*., 1998; Nakozono *et al*., 2003) and proteomic studies (Majeran *et al*., 2005) in the leaves of $C_4$ plants to give clues about the activities of the $C_3$ bundle sheath.

## Bundle sheath structure

### *Bundle sheath anatomy in leaves*

In the leaf veins of numerous angiosperms the vascular bundles are surrounded, in whole or in part, by a distinct bundle sheath comprising one or more layers of compact parenchyma cells (Metcalfe, 1979). The bundle sheaths of dicotyledonous leaves usually consist of cells elongated parallel with the course of the bundle and having walls as thin as those of the adjacent mesophyll [but note that, even in leaves of $C_4$ plants, bundle sheath cells do not have thicker cell walls (von Caemmerer and Furbank, 2003)]. The bundle sheaths extend to the end of the bundles and completely enclose the terminal tracheids.

In a few $C_4$ species, the functional bundle sheath can exist independently of the vasculature. These 'distinctive' cells appear as bundle sheath (Kranz)-like cells lying in the mesophyll, separate from the parenchyma sheath, as single cells or in small groups (Johnson and Brown, 1973; Hattersley, 1984; Dengler *et al*., 1996). These only occur in a few small panicoid tribes (e.g. *Arundinella* species), which are also unusual in having widely spaced longitudinal veins, in striking contrast to other $C_4$ grasses in which close vein spacing is a consistent feature (Dengler *et al*., 1996). Similarly, leaves of some plants possess a bundle sheath which extends laterally beneath the palisade mesophyll cells, independently of the vasculature (the paraveinal mesophyll, see below).

In many dicotyledons, cells similar to those of the bundle sheath extend towards the epidermis and are sometimes collenchymically thickened (Esau, 1965). Leaves of sunflower have such bundle sheath extensions (Wylie, 1952; McLendon, 1992) so that lateral diffusion of gases within the mesophyll is prevented. Such leaves are referred to as heterobaric. In contrast, leaves largely lacking bundle sheath extensions are termed homobaric (Terashima, 1992; Lawson and Morrison, 2006). Leaves exhibiting bundle sheath extensions predominate in deciduous plants, whereas those without are predominantly evergreen broadleaved foliage of warmer climates (McLendon, 1992; Pieruschka *et al*., 2006). Esau (1965) has suggested that bundle sheath extensions may be involved in conduction, particularly as there is an inverse correlation with vein frequency and distribution (see also Roth-Nebelsick *et al*., 2001). Bundle sheath extensions appear to be an adaptation to saving water and to protecting the mesophyll against water stress (Terashima, 1992). Pieruschka *et al*. (2006) suggest that homobaric leaves found in hotter climates may have evolved to increase water use efficiencies, by allowing lateral $CO_2$ movement. Bundle sheath extensions in heterobaric leaves are also likely to reduce the spread of disease, and to provide structural support because these leaves tend to be thinner and more easily damaged by wind (Roth-Nebelsick *et al*., 2001; Pieruschka *et al*., 2006). Bundle sheath extensions may also behave as windows, increasing light penetration into the internal layers of the mesophyll, thus compensating for the reduction of the photosynthetic capacity per unit leaf area caused by the non-photosynthetic extensions (Nikolopoulos *et al*., 2002).

### *Bundle sheath anatomy in relation to transport*

Many grasses have two cell layers surrounding the vascular bundles, while others have a single layer. The outer layer is designated the parenchyma sheath, and the inner layer, whose inner walls are often thickened, is designated the mestome sheath (Figs 1, 2). The members of the subfamily Pooideae have this thick-walled mestome sheath, while in the subfamily Panicoideae there is only a parenchyma bundle sheath (Arber, 1934). The lack of airspaces between bundle sheath cells and at the bundle sheath–mestome sheath cell interface suggests a functional symplastic association between the outer and inner bundle sheath. Esau (1965) considered the bundle sheath to be an endodermis, and in some mestome sheaths Casparian strips have been identified (van Fleet, 1950). However, Lersten (1997) concludes that the Casparian strip in stems and leaves occurs unevenly among major taxa. It is common in rhizomes and leaves of pteridophytes, it is absent from gymnosperm stems, but is found in the leaves of at least some conifers (Wu *et al*., 2003), and it occurs in stems of at least 30 mostly herbaceous angiosperm families, but is far less common in leaves, where it is mostly reported from petioles (Lersten, 1997). Of course, the presence either of a Casparian strip or of extensive suberization in the bundle sheath would restrict apoplastic transport of solutes. Fluorescent dyes have been used to chart the movements of water from the xylem through

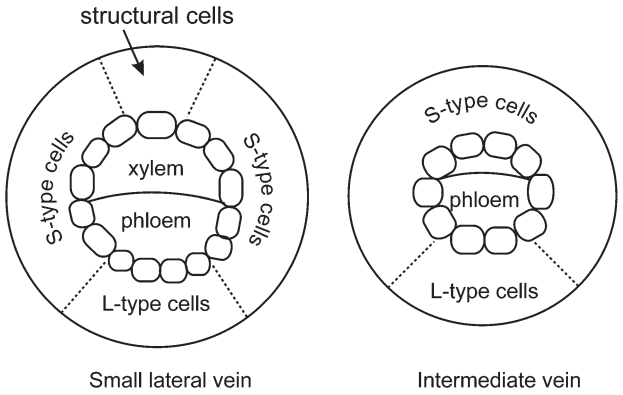


**Fig. 1.** Positioning of the S-type bundle sheath cell on the mestome sheath of barley leaves. Redrawn from Williams *et al.* (1989) with permission of Wiley-Blackwell.

some of the radial walls of mestome sheath cells near the xylem to the free space of the mesophyll (Peterson *et al.*, 1985; Canny, 1986). These authors have suggested that the suberized lamellae of the mestome sheath cells (O'Brien and Carr, 1970) must form an incomplete barrier near the xylem and permit apoplastic passage of dyes. Canny (1986) has hypothesized that suberized lamellae function to keep separate the two opposed fluxes through the sheath, the flux of water outwards and the flux of assimilates inwards, and they do this in two ways: first, by blocking parts of the walls of the mestome sheath or parenchyma sheath in such a way as to direct the water flux away from and around the phloem; and, secondly, by forming an insulation around the plasmodesmata in the pit membranes which keeps the apoplastic water movements remote from the symplast. In wheat, all of the longitudinal veins (though not transverse veins) are encased in a mestome sheath. All of the photosynthate that moves directly from the mesophyll to the sieve tubes in these veins must cross this boundary via the plasmodesmata that lie in the pit fields in the inner tangential wall (adjacent to the vascular tissue) of the mestome sheath cells (Kuo *et al.*, 1974). Within the wheat leaf as a whole, there is also evidence that different bundle types have different transport properties, with some bundle types having low sugar influxes and very high water effluxes and others having high sugar influxes and very low water effluxes. Thus these structures also keep these opposing flows separate (Kuo *et al.*, 1974).

Perhaps the best studied bundle sheath in $C_3$ plants is that of barley, in which the bundle sheath cells are large, vacuolate, and approximately cylindrical in shape, with a volume about four times that of mesophyll cells (Figs 1, 2). The majority of cells are termed S-type, containing small chloroplasts with approximately a third of the volume of mesophyll chloroplasts. In L-type bundle sheath cells, chloroplast volume and number per unit volume are similar to those of mesophyll cells. Structural cells are also present (Williams *et al.*, 1989). Williams *et al.* (1989) suggest that the L-type bundle sheath cells of barley may not be specialized for assimilate transport to the phloem, since their position does not favour direct unloading of

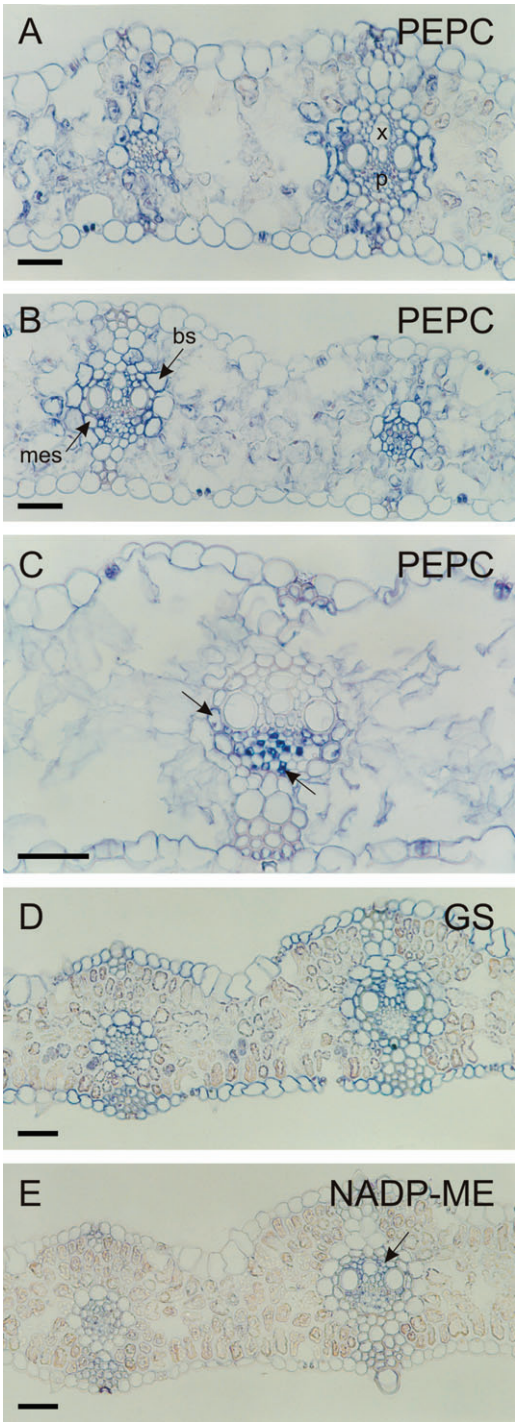


**Fig. 2.** Immunolocalization of PEP carboxylase (PEPC, A–C), glutamine synthetase (GS, D), and NADP-malic enzyme (NADP-ME, E) in barley leaves before (A, D, E) and after 4 d (B) or 8 d (C) dark senescence. bs, parenchymatous bundle sheath; mes, mestome sheath; p, phloem; x, xylem. Bar=50 μm. Micrographs were produced by LI Técsi. For details of methods, see Chen *et al.* (2000) and Famiani *et al.* (2000). For further explanation, see text.

assimilate to the mestome sheath (Fig. 1). This is because they abut an area of mestome sheath which has limited plasmodesmatal connections to the protophloem or adjacent mestome cells (Kuo *et al*., 1974; Altus and Canny, 1985). Williams *et al*. (1989) propose that the S-type bundle sheath cells play an important role in the transport of assimilate to the phloem via the mestome sheath, because they are ideally situated for sequestration of symplastic and apoplastic assimilate from both adaxial and abaxial sides of the leaf, and the large vacuole would permit considerable storage of sucrose and fructan. Williams *et al*. (1989) point out that the assimilate flux from the mesophyll to the phloem in $C_3$ leaves, such as barley, must be channelled through a smaller proportion of the total volume of the leaf than in $C_4$ grasses, since the total mesophyll cell/bundle sheath cell area ratio of transverse leaf sections of a range of $C_3$ grasses is relatively constant (~8.6), compared with between 1 and 4 in $C_4$ grasses (Hattersley, 1984).

### *How green is the bundle sheath?*

Many $C_3$ plants have chlorenchymatous bundle sheaths. The parenchyma sheath cells of some grasses contain chloroplasts, while the cells of other grasses do not (Rhoades and Carvalho, 1944). Of course, distinguishing between lack of chloroplasts and paucity of chloroplasts requires detailed analysis of many sections. In rice, there is considerable variation in the chloroplast density in the bundle sheath, not only between different wild rice species, but also within cultivated rice, with some bundle sheath cells possessing chloroplasts while others do not (Sheehy *et al*., 2007*a*). The presence of chloroplasts in the bundle sheath led Haberlandt (1914) to suggest that the green parenchyma sheath cells might have an undiscovered function in addition to their acting both as the efferent tissue and as an unimportant addition to the photosynthetic apparatus of the plant.

Crookston and Moss (1970) provide an extensive review of many plants with leaves reported to have chlorenchymatous sheaths. Most striking are plants with $C_4$ photosynthesis, which led Harberlandt (1914) to suggest the occurrence of co-operative photosynthesis in such plants. Downton and Tregunna (1968) reported that the concentration of chloroplasts in the specialized sheath in $C_4$ dicots was so great that these plants could be identified with the naked eye by detaching a leaf and viewing it against a light source. Crookston and Moss (1970) collected 88 dicotyledon species with chlorenchymatous vascular bundle sheaths, originating from 22 families. Of the 88 species, only three had low $CO_2$ compensation points and had bundle sheath chloroplasts that were specialized for starch formation (both features indicating that they were likely to possess the $C_4$ pathway). Whether or not the number of chloroplasts in the bundle sheath changes with developmental stage of the leaf, light environment, nutritional status, etc. is not known.

### *The paraveinal mesophyll (or extended bundle sheath)*

The leaves of many legume species contain a morphologically specialized tissue termed the paraveinal mesophyll (PVM) (Fisher, 1967; Franceschi and Giaquinta, 1983; Lansing and Franceschi, 2000) or Mittelschicht (middle layer) by Solederer (1908). The PVM comprises a horizontal layer of cells that form a reticulate, flattened network lying between the palisade parenchyma and spongy mesophyll. Kevecordes *et al*. (1987) showed that, in many cases, the PVM largely comprises bundle sheath cells and their long extensions, and therefore proposed the name extended bundle sheath (Kevecordes *et al*., 1987) (but not to be confused with bundle sheath extensions). The PVM is found in many legume species examined (Kevecordes *et al*., 1987; Lansing and Franceschi, 2000). Extended bundle sheath systems were also found in three of five non-legume species (Kevekordes *et al*., 1987) and in castor bean (Rutten *et al*., 2003). The PVM, with its large cells, lateral orientation, direct interface to the phloem region, and 10–20 or more palisade parenchyma cells contacting each PVM cell probably overcomes diffusion limitations imposed by multiple palisade layers. Studies on soybean PVM have shown that it plays a role in storage and partitioning of nitrogenous compounds, including temporary storage proteins (Franceschi and Giaquinta, 1983; Tranbarger *et al*., 1991). Microautoradiography of $^{14}CO_2$ pulse–chase studies in soybean has demonstrated that the PVM is an intermediary tissue in the transfer of photoassimilates moving from palisade parenchyma cells to the minor vein phloem (Lansing and Franceschi, 2000), a function earlier suggested by Haberlandt (1914) for such 'collecting cells'. PVM cells, but not mesophyll cells, were enriched in a sucrose-binding protein previously found to be associated with sucrose-transporting tissues (Lansing and Franceschi, 2000).

## Metabolic processes in the bundle sheath

### *Photosynthetic capacity*

Bundle sheath cells in barley are capable of photosynthesis, since bundle sheath cells can reduce tetrazolium blue, indicating photosystem II activity (Williams *et al*., 1989), can synthesize starch in the light, and single-cell immunoblotting from lateral bundle sheath cells shows that they contain amounts of Rubisco protein similar to those of single mesophyll cells (Koroleva *et al*., 2000). More sophisticated measurements in *Arabidopsis* have been used to measure changes in the quantum efficiency of photosynthetic electron transport, estimated from

chlorophyll fluorescence parameters that can be imaged from individual chloroplasts, in the bundle sheath and mesophyll cells of an intact leaf (Fryer *et al*., 2002). As pointed out above, the light environment within the bundle sheath may be affected by the occurrence of bundle sheath extensions, that may facilitate light penetration into the leaf (Nikolopoulos *et al*., 2002).

### *Carbohydrate synthesis*

A number of elegant techniques have been used to study the compartmentation of solutes and carbohydrate metabolism within the barley leaf, including single-cell sampling and analysis (Fricke *et al*., 1994; Koroleva *et al*., 1997), partitioning of recently fixed photoassimilates at single-cell resolution (Koroleva *et al*., 2000), immunolocalization (Koroleva *et al*., 2000), single-cell immunoblotting (Koroleva *et al*., 2000), and analysis of specific gene transcript abundance in individual plant cells (Gallagher *et al*., 2001; Koroleva *et al*., 2001).

The bundle sheath in barley appears to play a role in the rapid removal of assimilate from the apoplast and mesophyll cells, and in the continuous loading of the mestome sheath for phloem loading. It acts as a buffer for carbohydrates and malate between the mesophyll cells and the phloem, particularly when there is an excess of photosynthetic source capacity over sink demand (Koroleva *et al*., 2000). In barley leaves, sucrose and fructan accumulated in both mesophyll and bundle sheath cells during the photoperiod, but accumulation in the bundle sheath increased under conditions of reduced export of sugars from the leaves, due either to cooling of the roots and lower parts of the shoot or to increased photosynthesis under high light. The fructan: sucrose ratio was higher for bundle sheath cells than for mesophyll cells, suggesting that the threshold sucrose concentration needed for the initiation of fructan synthesis was lower for bundle sheath cells (Koroleva *et al*., 1998). There was a positive qualitative correlation between the presence of transcripts for an enzyme involved in fructan synthesis, sucrose: fructan 6-fructosyltransferase, and accumulation of fructan and sucrose in both mesophyll and bundle sheath cells (Koroleva *et al*., 2001). The concentration of malate also increased during the photoperiod in mesophyll and bundle sheath cells (Koroleva *et al*., 2000).

Starch also accumulates in the bundle sheath of barley (Williams *et al*., 1989). Only L-type bundle sheath cells contained visible starch grains at the end of an 8 h dark period, a further 4 h darkness being required for complete mobilization of starch. Starch deposition within S-type bundle sheath and structural cells was detectable after 4 h illumination but was only appreciable in leaves excised from the plant and illuminated for 9–12 h. Hence, the absence of starch within the S-type (and the structural) bundle sheath cells at the beginning of the photoperiod may not only be a function of the small size of the chloroplasts, which would accumulate less starch, but may also reflect the proximity of the phloem sink. In the bundle sheath of rice, chloroplasts accumulated large amounts of starch up to the late stages of leaf development, in contrast to mesophyll chloroplasts, suggesting that bundle sheath chloroplasts of rice seedlings are specialized for the accumulation and supply of storage starch (Miyake and Maeda, 1976).

In $C_4$ plants, the picture is similar. In leaves from a wide range of $C_4$ plants grown under normal light conditions, starch is present only in the chloroplasts of the bundle sheath cells, and not in the mesophyll cells (Lunn and Furbank, 1997), and the key enzymes of starch synthesis (ADP-glucose pyrophosphorylase, starch synthase, and branching enzyme) are located primarily in the bundle sheath cells of maize leaves (Downton and Hawker, 1973; Echeverria and Boyer, 1986; Spilatro and Preiss, 1987; Majeran *et al*., 2005).

### *Import and export of nitrogen*

Nitrogen assimilation and recycling are compartmentalized between the mesophyll and the vasculature, and are shifted between different cellular compartments within these two tissues during the transition from sink leaves to source leaves (Brugière *et al*., 2000; Kichey *et al*., 2005). The results of detailed localization studies in rice leaves by Yamaya and co-workers indicate that the isoenzymes of glutamine synthetase (GS) and glutamate synthase (GOGAT) have quite distinct functions (Tobin and Yamaya, 2001). NADH-GOGAT protein was found to accumulate in vascular parenchyma cells and the mestome sheath cells of developing young rice leaves (Hayakawa *et al*., 1994). NADH-GOGAT is probably important in the reutilization of glutamine in developing sink organs (Hayakawa *et al*., 1994). As in the majority of higher plants, two isoenzymes of GS are present in rice leaves, one in the chloroplast (GS2) and a second in the cytosol (GS1) (Kamachi *et al*., 1992). GS1 protein was detected in companion cells and vascular parenchyma cells of senescing leaf blades of rice (Kamachi *et al*., 1992; Sakurai *et al*., 1996). In barley leaves, GS has similar locations, being prominent in the bundle sheath, mestome sheath, and xylem parenchyma, as well as the epidermal cells, although this immunolocalization does not distinguish between isoforms (Fig. 2D). In the vascular tissue of the flag leaf of wheat, GS1 protein was present in the primary pit fields connecting the mestome sheath cells and the neighbouring parenchyma and vascular cells. The finding that cytosolic GS is present in these cellular connections suggests that there is an active transfer of nitrogenous metabolites, such as glutamine, through these cells (Kichey *et al*., 2005), a function probably also performed by GS1 during nitrogen remobilization in rice

plants, in which glutamine is the major form of reduced nitrogen exported in the phloem sap from the senescing leaf (Hayashi and Chino, 1990; Yamaya *et al*., 1992). GS1 may also be involved in assimilating ammonia released by phenylalanine ammonia lyase during lignin synthesis.

As far as the synthesis of nitrogenous compounds for export is concerned, there is clear evidence that in mature, non-senescent, leaves of spinach, barley, and sugar beet the phloem sap has a similar amino acid composition to the mesophyll sap, indicating that there is no amino acid metabolism between the mesophyll cells and the sieve tube, and that the overall process of transfer, although carrier-mediated, may be a passive process wholly dependent upon the mass flow of solutes driven by phloem loading (Riens *et al*., 1991; Winter *et al*., 1992; Lohaus *et al*., 1994). However, in melon (*Cucurbita melo*), diurnal fluctuations in amounts of individual amino acids in the phloem showed no distinct correlation with patterns seen in the leaf sap. For example, at midday, the mesophyll sap was enriched in aspartate, whereas the phloem sap was enriched in glutamine (Mitchell *et al*., 1992). This requires that the necessary metabolism of amino acids does not occur in the bulk of the leaf, but must occur within the vasculature or in the cells surrounding it. In addition, cucurbits possess a number of compounds which are specifically involved in transport, such as arginine and citrulline. The site of their synthesis is unknown, but it is conceivable that these compounds are made in or around the vasculature in cells adjacent to the site of export from the leaf.

In the leaves of $C_4$ plants, primary nitrogen assimilation occurs within the mesophyll (Rathnam and Edwards, 1976; Harel *et al*., 1977; Becker *et al*., 1993). However, the localization of GS and Fd-GOGAT involved in secondary nitrogen assimilation is less clear, perhaps due to differences in growth conditions between the various studies (Rathnam and Edwards, 1976; Harel *et al*., 1977; Becker *et al*., 1993, 2000). Recent proteomic studies of maize have led to quantification of four chloroplast enzymes (with one isoform each) involved in nitrogen metabolism (Majeran *et al*., 2005). Three enzymes predominantly expressed in the mesophyll chloroplasts were nitrate reductase (2.5–6-fold enrichment), Fd-GOGAT-1 (5-fold or unique in the mesophyll), and aspartate aminotransferase (2–11-fold) (see also Hatch and Mau, 1973). The bundle sheath to mesophyll ratio was close to 1 for GS (Majeran *et al*., 2005).

### *Sulphur assimilation*

The expression of three functional sulphate transporters (*Sultr1;1*, *Sultr2;1*, and *Sultr2;2*) in *Arabidopsis* has been studied by Takahashi *et al*. (2000), who suggest a direct role for the bundle sheath in sulphur transport. *Sultr1;1* was localized in the lateral root cap, root hairs, epidermis, and cortex of roots, while *Sultr2;1* was localized in the xylem parenchyma cells of roots and leaves, and in the root pericycles and leaf phloem. *Sultr2;2* showed specific localization in the root phloem and leaf bundle sheath cells. The latter suggests a role in the uptake of sulphate released from xylem vessels for transfer to the primary sites of assimilation in leaf mesophyll cells. The observed up-regulation of the expression of *Sultr2;2* in bundle sheath cells and down-regulation of the expression of *Sultr2;1* in leaf xylem parenchyma cells during sulphur stress could enhance uptake by bundle sheath cells in order to maintain a supply of sulphate to leaf cells for sulphur assimilation (Takahashi *et al*., 2000). Burgener *et al*. (1998) have shown that, in the $C_4$ plant, maize, assimilatory sulphate reduction is restricted to the bundle sheath cells, whereas the formation of glutathione takes place predominantly in the mesophyll cells, with cyst(e)ine functioning as a transport metabolite between the two cell types.

### *Is the bundle sheath pre-adapted for a role in $C_4$ photosynthesis?*

Hibberd and Quick (2002) have argued that the photosynthetic cells surrounding the vascular system are predisposed to $C_4$ photosynthesis because they are already enriched in key decarboxylases, such as malic enzyme (ME) and phosphoenolpyruvate carboxykinase (PEPCK). Hibberd and Quick (2002) compared the activities of three decarboxylation enzymes for C4 acids, NAD-ME, NADP-ME, and PEPCK, indicative of the three different $C_4$ subtypes, in the petiole and its surrounding photosynthetic cells with those in the leaf lamina. In cells surrounding and including the xylem and phloem of the tobacco petiole, the activity of NAD-ME was 13-fold higher than in the leaf, and the activities of NADP-ME and PEPCK were both 9-fold higher than in the leaf. However, this analysis did not further consider the compartmentation that might occur within the vascular bundle. Overall, the data suggest that the highest expression of PEP carboxylase, PEPCK, and NADP-ME does not occur in the same cells in $C_3$ plants as in the leaves of $C_4$ plants. For example, PEPCK is specifically located within the phloem companion cells in cucumber leaves, stems, and petioles (Chen *et al*., 2004), and is also present in the phloem in a number of other plants, including *Arabidopsis* (Malone *et al*., 2007). There is evidence that it participates in the metabolism of asparagine in transport tissues (Delgado-Alvarado *et al*., 2007).

Studies of the expression of the NADP-ME gene family in *Arabidopsis* have shown that the four genes encoding putative NADP-MEs (NADP-ME 1–3, which are cytosolic, and NADP-ME4, which is chloroplastic) all showed expression in or around the vasculature of leaves, stems,

and roots at various stages of development (Wheeler *et al*., 2005). However, in cucumber cotyledons (data not shown) and in barley leaves, NADP-ME is predominantly located within the xylem parenchyma (Fig. 2E, arrow), rather than in the bundle sheath. Wheeler *et al*. (2005) suggested that the presence of NADP-ME in the vasculature could be involved in controlling malate concentration, thus regulating the pH of the xylem and/or reflecting a role in providing NADPH for lignin biosynthesis.

Interestingly, PEP carboxylase, which is highly expressed in the mesophyll of $C_4$ plants (Stockhaus *et al*., 1997), appears to be more abundant in the bundle sheath of barley leaves (Fig. 2A). In non-senescent barley leaves, PEP carboxylase was present throughout the leaf, but was prominent in the stomata (Fig. 2A–C) and in the vasculature, particularly the lateral cells of the bundle sheath and mestome sheath adjoining the mesophyll and the phloem (see also Chen *et al*., 2000). Consistent with this observation, both PEP carboxylase and Rubisco proteins were found throughout the leaf chlorenchyma of tobacco leaves (Reed and Chollet, 1985), and the promoter region of the non-photosynthetic PEP carboxylase gene of *Flaveria pringlei* ($C_3$) induces reporter gene expression mainly in the vascular tissue of leaves and stems, as well as in mesophyll cells of transgenic *Flaveria bidentis* ($C_4$) plants (Stockhaus *et al*., 1997). In detached barley leaves incubated in the dark for 4 d, the predominant location of PEP carboxylase shifted during senescence to the lateral cells of the bundle sheath and the vasculature (Fig. 2B) and, by 8 d, was particularly prominent in the lateral cells of the mestome sheath and in the phloem (Fig. 2C, arrows), as also seen in senescing cucumber cotyledons (Chen *et al*., 2000). The presence of PEP carboxylase in these cells indicates an enhanced anaplerotic capacity which could be connected to enhanced export of organic or amino acids. The pattern of expression probably allows an efficient translocation of assimilates until the very late stages of leaf senescence (Brugière *et al*., 2000) and it also suggests that the roles of the bundle sheath and mestome sheath increase during senescence as they become a pivotal point in the export of nitrogen and other important nutrients from the leaf to the remainder of the plant.

### *$CO_2$, oxygen, and reactive oxygen species in the bundle sheath*

The lack of air spaces between bundle sheath cells (that prevent inward diffusion of $CO_2$) and the dense tissues of the vasculature [that may have a high respiratory demand for $O_2$ (van Bel and Knoblauch, 2000)], and the possibility that the vasculature may itself deliver malate that can be decarboxylated with the release of $CO_2$ (Hibberd and Quick, 2002) raises questions concerning the relative concentrations of these gases in the $C_3$ bundle sheath cells. Koroleva *et al*. (2000) consider that both mesophyll and bundle sheath cells of barley equilibrate rapidly with incoming $CO_2$, whereas Morison *et al*. (2005) suggest that atmospheric $CO_2$ is unlikely to exhibit significant rates of lateral diffusion from stomatal cavities to vascular tissues during photosynthesis, even in homobaric leaves.

As far as respiration is concerned, the majority of cytochrome oxidase protein in barley leaves was found in the mesophyll cytoplasm and only a small proportion was detected in the bundle sheath cells and in a few phloem parenchyma cells (Koroleva *et al*., 2000). In young wheat leaves, mesophyll cells contain 40% more mitochondria than vascular cells (Robertson *et al*., 1995). This indicates that the mesophyll cells have higher respiratory rates, which may perhaps reflect higher rates of photorespiration in these cells, either because of a higher intrinsic capacity for photorespiration compared with bundle sheath cells [the P subunit of glycine decarboxylase was less abundant in the bundle sheath chloroplasts than in the mesophyll chloroplasts of pea leaves (Tobin *et al*., 1991)] or because of lower rates of photorespiration in the $C_3$ bundle sheath caused by higher $CO_2$ and/or lower $O_2$ concentrations.

An indication of differences in oxygen concentration in the bundle sheath are the differences in the effects of anoxia on the rate of phloem loading in attached leaves of $C_3$ and $C_4$ monocots and dicots (Thorpe and Minchin, 1987). Phloem loading decreased under anoxia in $C_3$ leaves, but not in general in the leaves of either $C_4$ monocots or dicots in the light. However, the phloem loading rate in the $C_4$ leaves was reduced if the leaf was also darkened or received no $CO_2$. It was suggested that insensitivity to anoxia in $C_4$ leaves is due to photosynthetic $O_2$ evolution which leads to $O_2$ concentrations in the bundle sheath sufficient to energize a phloem-loading system (Thorpe and Minchin, 1987). Conversely, it implies that photosynthetic $O_2$ generation in at least some $C_3$ bundle sheaths is inadequate to overcome the effects of anoxia.

Generation of reactive oxygen species has been visualized in *Arabidopsis* leaves. Singlet oxygen has been detected by infiltrating leaves with dansyl-2,2,5,5,-tetramethyl-2,5-dihydro-1H-pyrrole (DanePy), a dual fluorescent and spin probe, and superoxide anion and $H_2O_2$ were detected using nitroblue tetrazolium and 3,3-diaminobenzidine (Fryer *et al*., 2002). *Arabidopsis* leaves respond to light stress by accumulating $H_2O_2$ specifically in the vascular bundles (Fryer *et al*., 2002, 2003), with the bundle sheath cell chloroplasts the major source of $H_2O_2$. Generation of $H_2O_2$ is dependent on photosynthetic electron transport, presumably as a result of the Mehler reaction (Fryer *et al*., 2003). Similarly, in tomato leaves, active oxygen species are generated near cell walls of vascular bundle cells in response to wounding (Orozco-Cárdenas *et al*., 2001). It may be that bundle sheath tissue synthesizes more $H_2O_2$

than mesophyll tissues, particularly if $CO_2$ availability were limiting as a result of slow diffusion of $CO_2$ from stomatal cavities to vascular tissues in photosynthesizing leaves, leading to an enhanced Mehler reaction (Morison *et al*., 2005).

*Arabidopsis* bundle sheath cells therefore differ from neighbouring leaf tissues in $H_2O_2$ and antioxidant metabolism. Cytosolic ascorbate peroxidase (*APX2*) is expressed specifically in bundle sheath cells (Fryer *et al*., 2003), and the accumulation of $H_2O_2$ in the chloroplasts of the bundle sheath parallels the induction of *APX2* expression (Fryer *et al*., 2003; Chang *et al*., 2004). Differences in antioxidant metabolism also exist in the $C_4$ plant, maize, and are indicated by the fact that glutathione reductase and dehydroascorbate reductase were almost exclusively localized in the leaf mesophyll tissue, whereas ascorbate, ascorbate peroxidase, and superoxide dismutase were largely absent from the mesophyll fraction. Catalase, reduced glutathione, and monodehydroascorbate reductase were found to be approximately equally distributed between the two cell types. It was concluded that the antioxidants in maize leaves are partitioned between the two cell types according to the availability of reducing power and that oxidized glutathione and dehydroascorbate produced in the bundle sheath tissues have to be transported to the mesophyll for re-reduction (Doulis *et al*., 1997).

Fryer *et al.* (2003) have suggested that $H_2O_2$ from bundle sheath cell chloroplasts may be part of a wider systemic signalling network. One *Arabidopsis* mutant, *alx8*, has constitutively higher *APX2* expression and higher contents of foliar abscisic acid (ABA) than the wild type. The responsiveness of *APX2* to exogenous ABA, the coincident increase in ABA and *APX2* gene expression in *alx8*, and the elevated ABA content in high-light-treated wild-type plants suggest a role for ABA in the network of transcriptional responses to high light (Rossel *et al*., 2006). Interestingly, there is evidence that certain steps in ABA biosynthesis are restricted to specific vascular tissues, as their tissue expression patterns are distinct from the expression patterns of other genes that are important for ABA biosynthesis (Cheng *et al*., 2002; Koiwia *et al*., 2004; Christmann *et al*., 2005). Thus sensing of water stress and generation of ABA may be located in close proximity (Christmann *et al*., 2005). Interestingly, in rice bundle sheath, chloroplasts appear to have a higher sensitivity to drought stress than mesophyll chloroplasts, as evidenced by a decreased content of Rubisco (Yamane *et al*., 2003).

## Conclusion

Although it is clear that the cells of the bundle sheath and their extensions have a number of metabolic roles, for example, in photosynthesis, synthesis and storage of carbohydrates, the uptake, metabolism, and mobilization of nitrogen and sulphur, and in antioxidant metabolism, it is clear that much more needs to be known about their activities in the leaves of $C_3$ plants. The bundle sheath is a critical control point for the supply of water and solutes to leaf cells (Fricke, 2002) and for the export of the same. If bundle sheath cells were capable of detecting such fluxes, they would seem a logical location for a flux sensor. Knowledge of the activities of the $C_3$ bundle sheath is also important in relation to attempts to engineer $C_4$ photosynthesis into $C_3$ crops, which would result in large changes to the bundle sheath cells of the $C_3$ target species. These changes might include additions of various enzymes and transporters to the chloroplast and cytosol, changes in cell wall permeability, and increases in chloroplast number (Sheehy *et al*., 2007*b*). Since, unlike the bundle sheath of $C_4$ plants, mechanical purification of these bundle sheath cells from $C_3$ leaves is impossible, at least from spinach leaves (Huang and Beevers, 1972), most studies have employed techniques such as *in situ* hybridization, immunohistochemistry, reporter gene expression (often without regard to compartmentation within the vasculature), or single-cell sampling. However, there are now advances in techniques for studying gene expression and modulating it in specific cell types. In addition, chloroplasts can be isolated from individual cell types by expressing yellow fluorescent protein on their outer surface, and then isolating them immunogenically (Truernit and Hibberd, 2007). Such techniques can be expected to lead to an improved understanding of the physiological roles of the bundle sheath in leaves.

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