On the mechanism of C₄ photosynthesis intermediate exchange between Kranz mesophyll and bundle sheath cells in grasses

Paweł Sowiński¹, Jarosław Szczepanik¹ and Peter E. H. Minchin³

¹ University of Warsaw, Institute of Plant Experimental Biology, Department of Plant Growth and Development, Miecznikowa 1, 02-096 Warszawa, Poland
² Plant Breeding and Acclimatization Institute, Plant Biochemistry and Physiology Department, Radzików, 05-870 Błonie, Poland
³ The Horticulture and Food Research Institute of New Zealand Ltd, 412 No 1 Road, RD 2 Te Puke 3182, New Zealand

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Abstract

C₄ photosynthesis involves cell-to-cell exchange of photosynthetic intermediates between the Kranz mesophyll (KMS) and bundle sheath (BS) cells. This was believed to occur by simple diffusion through plentiful plasmodesmatal (PD) connections between these cell types. The model of C₄ intermediates’ transport was elaborated over 30 years ago and was based on experimental data derived from measurements at the time. The model assumed that plasmodesmata occupied about 3% of the interface between the KMS and BS cells and that the plasmodesmata structure did not restrict metabolite movement. Recent advances in the knowledge of plasmodesmatal structure put these assumptions into doubt, so a new model is presented here taking the new anatomical details into account. If one assumes simple diffusion as the sole driving force, then calculations based on the experimental data obtained for C₄ grasses show that the gradients expected of C₄ intermediates between KMS and BS cells are about three orders of magnitude higher than experimentally estimated. In addition, if one takes into account that the plasmodesmata microchannel diameter might constrict the movement of C₄ intermediates of comparable Stokes’ radii, the differences in concentration of photosynthetic intermediates between KMS and BS cells should be further increased. We believe that simple diffusion-driven transport of C₄ intermediates between KMS and BS cells through the plasmodesmatal microchannels is not adequate to explain the C₄ metabolite exchange during C₄ photosynthesis. Alternative mechanisms are proposed, involving the participation of desmotubule and/or active mechanisms as either apoplastic or vesicular transport.

Key words: C₄ photosynthesis, grasses, modelling, plasmodesmata, symplasmic transport.

C₄ photosynthesis

The C₄ carbon cycle involved in carbon dioxide trapping prior to photosynthesis has been well researched since its discovery in the late 1960s. This process involves morphological and physiological adaptations, so it has been studied by anatomists, biochemists, and physiologists. This pathway enables carbon dioxide to be concentrated at the site of Rubisco action, reducing photorespiration and enhancing water use efficiency.

Primary carbon assimilation (PCA) takes place in the Kranz mesophyll (KMS) cells. The product of phosphoenolpyruvate (PEP) carboxylation, i.e. oxaloacetate is converted to either malate or aspartate. C₄ acids are exported to the bundle sheath (BS) cells where they are decarboxylated. The released CO₂ is incorporated into the Calvin cycle for primary carbon reduction (PCR). The route of decarboxylation depends on the sub-type of C₄ photosynthesis: NADP-malic enzyme (NADP-ME),...
NAD-malic enzyme (NAD-ME), and PEP-carboxykinase (PEP-CK). After reduction, a fraction of the assimilated carbon moves back from the BS to the KMS cells as pyruvate, where it is regenerated into PEP. Phosphoglyceride (PGA) and triosephosphate (TP) are also shuttled to the KMS (Furbank and Foyer, 1988).

The architectural arrangement of the cells involved in photosynthesis and photosynthetic export optimizes this cell-to-cell exchange. According to Gamalei’s (1991) classification based upon the route of phloem loading, the veins in C4 plants represent a type 2c ultrastructure, specific for many C4 and crassulacean acid plants. In plants with this vein ultrastructure type, Kranz mesophyll layer(s) surround the bundle sheath layer, and are interconnected by numerous plasmodesmata, while the number of plasmodesmata between companion cell/sieve tube complex and adjoining cells is limited.

In C4 grasses, symplasmic continuity exists between the Kranz mesophyll, the bundle sheath, and the vascular parenchyma (VP). In some species (Botha, 1992) or subspecies (Sowiński et al., 2001), symplasmic continuity occurs between bundle sheath cells and companion cells, but this is rare. In grasses, sieve tubes in small and intermediate vascular bundles are of two types: thin-walled sieve tubes connected to companion cells, and thick-walled sieve tubes connected to vascular parenchyma cells. The role of the thick-walled sieve tubes is still unknown, while the companion cell/thin-walled sieve tube complex is responsible for phloem loading (Fritz et al., 1983). There are some anatomical differences among C4 photosynthesis sub-types, manifested mostly in the distribution of BS chloroplasts, located centrifugally in NADP-ME, PEP-CK, and PCK-like NAD-ME species and centripetally in the classical NAD-ME species (Ohsugi and Murata, 1986; Dengler et al., 1994; Giussani et al., 2001; Ueno et al., 2006). There is general agreement that exchange of C4 photosynthetic intermediates between KMS and BS cells is solely through plasmodesmata (Hattersley and Browning, 1981; Hattersley, 1987, but see Eastman et al., 1988a, b). The role of plasmodesmata in C4 photosynthesis is supported by the positive correlation between the number of plasmodesmata and the net photosynthesis rate found in several C4 grasses (Botha, 1992; Sowiński et al., 2007). In species that synthesize sucrose in KMS, it is symplastically transported through at least three cells: KMS–BSC–VP, before being loaded into the phloem. The crucial role of plasmodesmata in the export of photosynthates from leaves finds strong support in studies of a maize mutant, SXD-1 (Russin et al., 1996), in which plasmodesmata at the BSC/VP interface were occluded by callose (Botha et al., 2000), resulting in the arrest of sucrose export. All these data support the conclusion that the rates of C4 photosynthesis and photosynthetic export depend on the number and conductivity of plasmodesmata.

Plasmodesmata linking KMS and BS cells in C4 grasses differ in ultrastructure and dimensions (Botha et al., 2005, and literature cited herein). In some species, sphincters may occur on one or both cell sides (Evert et al., 1977; Robinson-Beers and Evert, 1991; Botha et al., 2005). KMS/BS plasmodesmata diameter is of approximately 100 nm, however, if suberin lamellae are present plasmodesmata diameter might be restricted down to approximately 40 nm (Robinson-Beers and Evert, 1991; Botha et al., 2005). Even if plasmodesmata do not cross suberin lamellae (NAD-ME sub-type), they show constriction at the neck regions down to approximately 40 nm (Valle et al., 1989; Sowiński et al., 2007). The diameter of plasmodesmata at the KMS/BS interface in the dicotyledonous C4 plant Salsola kali L. was approximately 50 nm (Olesen, 1975).

**Mechanism of C4 intermediate transport between KMS and BS cells**

It has been proposed that C4 photosynthesis intermediates were transported between KMS and BS cells by means of diffusion, driven by a concentration gradient (Leegood, 2000, and citations therein). This was supported by estimations of concentration differences of the main photosynthetic metabolites in maize (Leegood, 1985; Stitt and Heldt, 1985) that were in agreement with values obtained by modelling transport of the C4 intermediates (Osmond, 1970; Hatch and Osmond, 1976). The model, elaborated over 30 years ago, was based on the experimental data of Tyree (1970). Authors assumed that plasmodesmata occupied about 3% of the interface between the KMS and BS cells and that the plasmodesmata structure did not constrict metabolic movement. Recent advances in knowledge of plasmodesmatal structure throw doubt on these assumptions, so these are revised, taking into account the new anatomical details.

The number of plasmodesmata linking KMS and BS cells in C4 plants is well documented (Botha, 1992; Cooke et al., 1996; Sowiński et al., 2007) and it is agreed that this number is higher in C4 than in C3 plants (Botha, 1992; Cooke et al., 1996), with C4 plants having approximately 6 plasmodesmata μm−2 of KMS/BS interface (Table 1). With a plasmodesma diameter of 40 nm, the total plasmodesmatal cross-section occupies approximately 0.8% of the cellular interfaces. However, according to present knowledge of plasmodesmatal ultrastructure, part of the cross-section is occupied by the desmotubule and transport takes place within the 7–9 microchannels (Overall et al., 1982; Ding et al., 1992), each with a diameter of 2.5–4 nm (Overall et al., 1982; Roberts and Oparka, 2003). Therefore the cross-section open for transport would constitute only ~0.07% of total KMS/BS interface area, i.e. two orders of magnitude less than is assumed for models postulated 30 years ago. With this limitation on the area available for exchange, simple
diffusion would not seem to be sufficient to account for the volume of metabolites being transported. This problem has led us to propose new calculations. Our calculations are based on the experimental data obtained for C₄ grasses, representing all three C₄ sub-types (Botha et al., 1982; Ohsugi and Murata, 1986; Botha and Evert, 1988; Valle et al., 1989; Botha, 1992; Soros and Dengler, 1998; Ueno et al., 2006; Sowiński et al., 2007) and show that the expected gradients between KMS and BS cells of C₄ intermediates are much higher than experimentally estimated. These calculations confirm that diffusion-driven transport of C₄ intermediates between KMS and BS cells through the plasmodesmatal microchannels is not adequate to explain the observed concentration differences. An alternative mechanism is proposed.

**Simple diffusion: first approximation**

Diffusion through the plasmodesmatal microchannels in the cell wall can be treated as diffusion within a porous membrane, with microchannels acting as the pores. Then, the transport rate through such membranes will be affected in two ways: by the frequency of pores in a membrane and by the pore size. The importance of the porosity factor on the diffusion coefficient is rather obvious—the more pores within a membrane, the larger the space for diffusion (Bret-Harte and Silk, 1994; Patrick, 1997).

We are aware that plasmodesmatal microchannels are not simple tubes, but complex and irregular structures with many fjord-like structures branching out from the channel’s lumen. Such channel architecture might be thought to impede metabolite flux. However, while surface roughness does affect diffusivity of a single molecule, it has no effect on transport diffusivity. This difference is of great significance when the channel is rough even at the molecular level (Malek and Coppens, 2003), as in plasmodesmatal microchannels, which have diameters similar to the size of the transported metabolites (the diameters of photosynthesis intermediates are calculated further).
The other factor concerning plasmodesma architecture is its possible helical arrangement (Overall et al., 1982; Ding et al., 1992; Roberts, 2005). Diffusion will be most efficient, if transport channels are straight cylinders. If they are tortuous, the diffusion pathway inside a channel will increase. This may be the case of plasmodesmatal microchannels. Anyway, without detailed knowledge on plasmodesmata ultrastructure, a helical arrangement of microchannels could not be taken into account.

Assumptions for the model
Assuming that diffusion is the only mechanism involved in transport of metabolites between KMS and BS cells and that this is through the microchannels of the plasmodesmata, then there must be a sufficient concentration gradient of each metabolite to sustain diffusion flow given by Fick’s law:

\[ J = -D \nabla c \]  

where \( \nabla c = \frac{\partial c}{\partial x} \) denotes concentration gradient, and \( D \) is the diffusion coefficient for the specific metabolite.

To calculate the concentration gradient necessary to sustain diffusion between KMS and BS cells, we start with several assumptions, most of them intentionally chosen as favourable for diffusion. The assumptions are shown in Table 1.

Biometric data and cross-section of transport channels
To calculate the symplasmic flow of photosynthates between KMS and BS cells, experimental data of six C4 grasses have been considered: Zea mays (NADP-ME), Digitaria sanguinalis (NADP-ME), Themeda triandra (NADP-ME), Panicum miliaceum (classical NAD-ME), Eragrostis plana (classical NAD-ME), and Panicum maximum (PEP-CK). Panicum miliaceum and Eragrostis plana will be further referred to as NAD-ME species. All biometric and carbon flux data (Table 2) have been taken, or calculated, from published data (Botha et al., 1982; Oshugi and Murata, 1986; Botha, 1992; Soros and Dengler, 1998; Ogle, 2003; Ueno et al., 2006; Sowiński et al., 2007).

The area of KMS/BS cell walls (\( S_W \)) mm\(^{-2}\) of leaf area was calculated as:

\[ S_W = \frac{1000}{I_{BS}} C_{BS} n_v \]  

where 1000 is a conversion factor, since 1 mm = 1000 \( \mu m \), \( I_{BS} \) is equal to 0.5 and allows for the contribution of intercellular spaces to BS circumference (Table 1), \( C_{BS} \) is a circumference of BS cells (Table 2), \( n_v \) gives the number of veins in leaf segment of 1 mm\(^2\) (Table 3).

Total cross-sectional area of microchannels (\( T_{SK} \)) is given by:

\[ T_{SK} = 9 f_{PD} S_k \]  

for nine microchannels per plasmodesma (Table 1), \( f_{PD} \) is the number of plasmodesmata mm\(^{-2}\) of leaf area (Table 3), and \( S_k \) is the cross-sectional area of a single microchannel (\( S_k = 12.56 \, \text{mm}^2 \)).

On the basis of equations (2) and (3), \( \varphi = T_{SK}/S_W \) is defined as the surface fraction of plasmodesmata microchannels in the KMS/BS cell walls mm\(^{-2}\) of leaf area. The results of calculations of \( \varphi \) made for different C4 photosynthesis sub-types are shown in Table 3. The surface fraction of microchannels in NADP-ME and PCK sub-types is just about 0.06% and in NAD-ME species having the highest \( \varphi \) values, it is only 0.3%.

Diffusion coefficients for transported metabolites
Diffusion coefficients in water for each metabolite were calculated using the Stokes–Einstein formula:

\[ D(i) = \frac{kT}{6\pi \eta r_{ST}} \]  

where \( D(i) \) is the diffusion coefficient for metabolite \( i \) of Stokes’ radius \( r_{ST} \) in solution of viscosity \( \eta \) and temperature \( T = 298.15 \, \text{K} \) and \( k = \)Boltzman’s constant (1.38\( \times 10^{-23} \, \text{J} \, \text{K}^{-1} \)).

Table 2. CO\(_2\) assimilation rates and biometric parameters for different C\(_4\) photosynthetic sub-types in grasses

<table>
<thead>
<tr>
<th>Photosynthetic sub-type</th>
<th>NADP-ME(^a)</th>
<th>NAD-ME(^a)</th>
<th>PEPCK(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO(_2) assimilation (( \mu)mol m(^{-2}) s(^{-1}))</td>
<td>13.00–23.00</td>
<td>23.00–27.00</td>
<td>22.00–23.00</td>
</tr>
<tr>
<td>(19.30) a, b</td>
<td>(25.00) a, b</td>
<td>(22.50) a, b</td>
<td></td>
</tr>
<tr>
<td>IVD, Intereavele distance (( \mu)m)</td>
<td>86.30–123.60</td>
<td>149.79–213.60</td>
<td>116.61–148.20</td>
</tr>
<tr>
<td>(109.88) a, b, c, d, e, f, g</td>
<td>(171.76) a, b, c, d, e, f</td>
<td>(131.75) a, b, c, d, e</td>
<td></td>
</tr>
<tr>
<td>C(_{BS}), Circumference of BS cells (( \mu)m)</td>
<td>153.20–211.50</td>
<td>240.40–246.70</td>
<td>263.80–292.70</td>
</tr>
<tr>
<td>(182.29) a, b, g</td>
<td>(243.55) a, b</td>
<td>(278.25) a, b</td>
<td></td>
</tr>
<tr>
<td>( r_{PD} ), number of PD per ( \mu)m of vein (( \mu)m(^{-1}))</td>
<td>284.92–559.00</td>
<td>2587.35–3045.00</td>
<td>574.00–857.41</td>
</tr>
<tr>
<td>(440.31) a, b</td>
<td>(2816.17) a, b</td>
<td>(715.70) a, b</td>
<td></td>
</tr>
<tr>
<td>PD per ( \mu)m(^2) KMS/BS interface (( \mu)m(^{-2}))</td>
<td>3.13–6.23</td>
<td>20.97–25.33</td>
<td>3.92–6.53</td>
</tr>
<tr>
<td>(4.74) a, b, h</td>
<td>(25.00) a, b</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) (a) Data from Sowiński et al. (2007); (b) data from Botha (1992); (c) calculated from Ogle (2003); (d) values from Ohsugi and Murata (1986); (e) data from Ueno et al. (2006); (f) data from Soros and Dengler (1998); (g) calculated from Botha et al. (1982); (h) data from Cooke et al. (1996).
Stokes’ radii for transported metabolites (Table 2) were determined using HyperChem 7.5 Student software (www.HyperChem.com), with all metabolites assumed to have no hydration spheres around them. The results of calculations are shown in Table 4.

There is no agreement on the viscosity of the cytoplasm. The mobility of BCECF (fluorescein derivate, MW 520) in cytoplasm using spot photobleaching was a quarter of that in water (Verkman, 2002), while in vivo measurements of GFP (27 kDa) movement in Escherichia coli was one-tenth of that in water (Sear, 2005, and references therein). However, in our calculations, the lowest reported value (1.2 mPa s) was used for the viscosity of the cytoplasm’s aqueous phase (Fushimi and Verkman, 1991).

The calculated diffusion coefficients for all considered metabolites are given in Table 4. The calculated data are comparable to values assumed by other authors (Hatch and Osmond, 1976).

Metabolite fluxes and concentration differences required to sustain diffusion between KMS and BS cells

The stoichiometry between carbon assimilation and C₄ metabolites transported between KMS and BS cells is shown in Fig. 1. All the assimilated CO₂ was assumed to be built into triose phosphates (C₃-P), which in turn were completely used for sucrose synthesis. It was assumed that for NADP-ME species sucrose was synthesized in KMS cells only, while for NAD-ME and PEP-CK species only half the sucrose was produced in KMS cells (Ohsugi and Huber, 1987; Usuda and Edwards, 1980). For all species examined, 60% of synthesized sucrose was assumed to be exported to the phloem (Sowiński et al., 2007).

Metabolite fluxes (J) were expressed here as a number of a given metabolite molecules [nM, (moles)] transported through 1 mm² of single channel’s cross-section (S_K=12.56 nm²) in 1 s, using the following equation:

\[
J = \frac{n_M}{9\pi \rho PD S_K}
\]

Calculated metabolite fluxes are given in Table 5.

The required concentration differences (\(\partial c\)) between KMS and BS cells to give the estimated flow rates for each metabolite was calculated using the transformed equation (1):

\[
\partial c = -J \frac{\partial x}{D}
\]

where J is given by equation (5), \(\partial x\) equals length of plasmodesma (150 nm, Table 1), and D is the

Table 3. Biometric parameters used in the model, calculated on the basis of mean values from Table 2

<table>
<thead>
<tr>
<th>Parameter</th>
<th>NADP-ME</th>
<th>NAD-ME</th>
<th>PEPCK</th>
<th>Calculation formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>(n_v), number of veins in 1 mm² of leaf blade (mm⁻²)</td>
<td>9.10</td>
<td>5.82</td>
<td>7.59</td>
<td>(n_v=1000n_M)</td>
</tr>
<tr>
<td>(f_{PD}), number of KMS/BS PD in 1 mm² of leaf blade (mm⁻²)</td>
<td>4.01×10⁶</td>
<td>16.39×10⁶</td>
<td>5.43×10⁶</td>
<td>(f_{PD}=1000n_v\rho PD)</td>
</tr>
<tr>
<td>(T_{S_K}), total cross-sectional area of plasmodesmal microchannels in cell walls between Kranz mesophyll (KMS) and bundle sheath (BS) cells (nm²)</td>
<td>0.45×10⁹</td>
<td>1.85×10⁹</td>
<td>0.61×10⁹</td>
<td>(T_{S_K}=9\rho PD S_K^2)</td>
</tr>
<tr>
<td>(S_W), total area of KMS/BS cell walls (nm²)</td>
<td>0.83×10¹²</td>
<td>0.71×10¹²</td>
<td>1.06×10¹²</td>
<td>(S_W=1000\rho BS C_{BS} n_v^6)</td>
</tr>
<tr>
<td>(\varphi), surface fraction of microchannels in KMS/BS cell walls of 1 mm² leaf blade</td>
<td>0.55×10⁻³</td>
<td>2.62×10⁻³</td>
<td>0.58×10⁻³</td>
<td>(\frac{S_K}{S_{S_K}})</td>
</tr>
</tbody>
</table>

*a* \(S_K\) area of single microchannel’s cross-section (12.56 nm²).

*b* See text for details.

Table 4. Molecular size, diffusion coefficients and confinement factors for metabolites transported between Kranz mesophyll and bundle sheath cells

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>(r_{ST}), Stokes’ radius (nm)</th>
<th>(D_t), diffusion coefficient in water (m² s⁻¹)</th>
<th>(D_{cyt}), diffusion coefficient in cytoplasm (m² s⁻¹)</th>
<th>Confinement factor inside microchannel with diameter of 4 nm ((K_c))</th>
<th>Confinement factor ((K_c)) inside desmotubule of diameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malate</td>
<td>0.27</td>
<td>8.12×10⁻¹⁰</td>
<td>6.77×10⁻¹⁰</td>
<td>0.56</td>
<td>0.86</td>
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<td></td>
<td></td>
<td>0.92</td>
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<td></td>
<td></td>
<td>0.94</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>0.26</td>
<td>8.40×10⁻¹⁰</td>
<td>7.00×10⁻¹⁰</td>
<td>0.57</td>
<td>0.87</td>
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<td>0.92</td>
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<td>0.94</td>
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<tr>
<td>Alanine</td>
<td>0.26</td>
<td>8.40×10⁻¹⁰</td>
<td>7.00×10⁻¹⁰</td>
<td>0.57</td>
<td>0.87</td>
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<td>0.92</td>
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<td></td>
<td></td>
<td>0.94</td>
</tr>
<tr>
<td>Aspartate</td>
<td>0.34</td>
<td>6.40×10⁻¹⁰</td>
<td>5.33×10⁻¹⁰</td>
<td>0.47</td>
<td>0.83</td>
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<td>0.89</td>
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<td></td>
<td></td>
<td></td>
<td>0.92</td>
</tr>
<tr>
<td>PEP</td>
<td>0.35</td>
<td>6.30×10⁻¹⁰</td>
<td>5.25×10⁻¹⁰</td>
<td>0.47</td>
<td>0.83</td>
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<td>0.89</td>
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<td>0.92</td>
</tr>
<tr>
<td>C₃-P</td>
<td>0.35</td>
<td>6.30×10⁻¹⁰</td>
<td>5.25×10⁻¹⁰</td>
<td>0.47</td>
<td>0.82</td>
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<td>0.89</td>
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<td></td>
<td>0.92</td>
</tr>
<tr>
<td>Sucrose</td>
<td>0.44</td>
<td>5.00×10⁻¹⁰</td>
<td>4.17×10⁻¹⁰</td>
<td>0.38</td>
<td>0.78</td>
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<td>0.87</td>
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<td>0.90</td>
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</table>

*a* Assuming 1.2 times higher viscosity for cytoplasm than for water (Fushimi and Verkman, 1991).
plasmodesmatal diffusion coefficient ($D_{PD}$) taken to be proportional to the surface fraction of microchannels ($\varphi$, Table 3) in KMS/BS cell walls for a leaf segment of 1 mm$^2$ and cytoplasmatic diffusion coefficient ($D_{cyt}$, Table 4):

$$D_{PD}(i) = D_{cyt}(i) \varphi$$  (7)

The values of $\partial c$ obtained in our model are shown in Table 5. The calculated data have been compared with experimental data obtained by Stitt and Heldt (1985). Their data concerned concentrations of C$_4$ intermediates in KMS and BS cells of maize. These authors obtained concentrations of C$_4$ metabolites as high as a few hundreds of nanomoles per mg of chlorophyll (mg Chl). They assumed that the chlorophyll was equally distributed between KMS and BS cells and that the combined volume of chloroplasts and cytoplasm was 40 $\mu$m per mg Chl. As a result, the estimated concentration of each metabolite between KMS and BS cells was a few nanomoles per $\mu$m, equal to a few milimoles per litre. Comparison of the data of Stitt and Heldt (1985) with the values of $\partial c$ we calculated using the different approaches, are shown in Table 3.

In addition, the values of $\partial c$ proposed by Weiner et al. (1988) are shown in Table 5. Authors assumed a concentration gradient of 1 mM to describe the rate of diffusion of particular photosynthetic metabolite into BS cells.

Calculated concentration differences of metabolites required to sustain diffusion are higher by about three orders of magnitude, as compared to experimental data (Stitt and Heldt, 1985; Weiner et al., 1988). Moreover, differences between photosynthetic types were observed: in NAD-ME species, concentration differences were about three times lower than in NADP-ME and PEP-CK species, characterized by similar values of $\partial c$. These discrepancies reflect different plasmodesmatal frequency (Table 2).

Conclusions to the simple diffusion approximation

The estimated concentration differences required assuming transport by diffusion through microchannels, were very high. These concentration differences, being tens of moles, seem unrealistic given that in the species studied to date, concentrations of C$_4$ metabolites and triose phosphates were in the order of a few tens of millimoles (Hatch and Osmond, 1976; Leegood, 1985, 2000; Stitt and Heldt, 1985). Similar discrepancies were noticed by Bret-Harte and Silk (1994), when they estimated solute deposition rates and corresponding fluxes in growing root of Zea mays, assuming that diffusion was the only mechanism for metabolite transport. Diffusion coefficients and concentration gradients calculated by these authors were a few orders of magnitude higher than expected. Our calculations of $D_{PD}$ and $\partial c$ made using Bret-Harte and Silk’s model gave values similar to the approach assuming transport through microchannels (data not shown).

Simple diffusion: second approximation

All C$_4$ metabolites considered were of similar size and therefore had similar diffusion coefficients (Table 4). All had low molecular weight, compared with the plasmodesmata exclusion limit of about 0.9 kDa, but their Stokes’ radii ($r_{ST}$) were quite high compared with the microchannel radius ($r_K=2$ nm). This observation raises queries of our assumption (Table 1) that microchannel diameter does not affect metabolite movement.
The dependence of diffusion coefficient on pore diameter is not simple. If the pore diameter is over ten times that of the transported molecule, then diffusion through the pore will equal that of the bulk fluid, while with a pore diameter smaller than two molecular diameters, single file diffusion will occur (Cui, 2005). For a ratio of pore width to molecular diameter of 2–10 (relevant to the metabolites we considered, where this was 4.5 to 7.69) pore diffusivity falls between those two extremes (Liu et al., 2005). Taking this into account, transport through a microchannel is the result of at least three types of diffusion: continuous diffusion (diffusion in bulk fluid), Knudsen’s diffusion, and surface diffusion, the latter two reducing transport (Gudmundsson, 2003; Valiullin et al., 2004; Liu et al., 2005). However, for the simplicity of current approximation, the diffusion through the microchannels was assumed to be continuous, and to vary with the ratio of the channel’s diameter to the molecular size, according to the confinement factor ($K_c$), defined as:

$$K_c = \left(1 - \frac{r_M}{r_K}\right)^4$$

(8)

which is a simplification of Renkin’s (1954) approach, valid for $r_{ST}/r_K > 0.01$. Equation (7) is now generalized to:

$$D_{PD}(i) = D_{cyt}(i)\phi K_c$$

(9)

The confinement factor varied from 0.38 to 0.54 (Table 4), results in a 2–3-fold slow down of diffusion inside the microchannel in relation to bulk fluid conditions, and correspondingly in approximately twice higher concentration differences of photosynthetic intermediates between KMS and BS cells necessary to sustain the transport of photosynthates between KMS and BS cells, as compared to the data obtained in the first approximation (Table 5).

**Simple diffusion model: the need for the third approximation?**

The model presented here is a highly simplified version of the situation encountered in planta. However, it shows, that even under assumptions favouring diffusion, the concentration differences of transported metabolites between KMS and BS cells necessary for maintaining the current net photosynthetic rates are high and hardly possible in living cells. If this model is to be valid in describing transport processes in vivo, several additional assumptions, neglected here, must be taken into consideration. The most important constriction to the model is that C₄ photosynthesis, because of its nature, needs exchange of metabolites between cells, i.e. simultaneous movement of some intermediates from KMS to BS, and others from BS to KMS. As it is stated above, Stokes’ radii of photosynthetic metabolites are comparable to the microchannel radius. So the assumption (Table 1), that two streams of molecules moving in opposite directions in narrow channels do not disturb each other, is improbable. In addition, transport of other compounds simultaneously with the transport of photosynthetic intermediates; the existence of hydration spheres around polar molecules

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Table 5. Metabolite fluxes ($mol \ mm^{-2} \ s^{-1}$) and concentration differences of photosynthetic metabolites ($mol \ dm^{-3}$) between Kranz mesophyll and bundle sheath cells in C₄ grasses

<table>
<thead>
<tr>
<th>Photosynthetic sub-type</th>
<th>C₄ metabolite</th>
<th>Metabolite fluxes through single microchannel</th>
<th>Concentration differences of metabolites between Kranz mesophyll and bundle sheath cells</th>
<th>Experimental data</th>
<th>No constrictions from channel size, all metabolites move through plasmodesmatal microchannels</th>
<th>Constrictions from channel size present, all metabolites move through plasmodesmatal microchannels</th>
<th>Metabolites passing from KMS to BS move inside desmotubule of diameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADP-ME</td>
<td>Malate</td>
<td>$4.26 \times 10^{-20}$</td>
<td>$0.018^a$</td>
<td>17.16</td>
<td>30.64</td>
<td>8.20</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>Pyruvate</td>
<td>$4.26 \times 10^{-20}$</td>
<td>$0.005^a$</td>
<td>17.16</td>
<td>30.10</td>
<td>30.10</td>
<td>30.10</td>
</tr>
<tr>
<td></td>
<td>C₃-P</td>
<td>$1.42 \times 10^{-20}$</td>
<td>$0.010^a$</td>
<td>7.51</td>
<td>15.98</td>
<td>15.98</td>
<td>15.98</td>
</tr>
<tr>
<td></td>
<td>Sucrose</td>
<td>$0.21 \times 10^{-20}$</td>
<td></td>
<td>1.40</td>
<td>3.68</td>
<td>0.73</td>
<td>0.09</td>
</tr>
<tr>
<td>NAD-ME</td>
<td>Alanine</td>
<td>$1.35 \times 10^{-20}$</td>
<td>$0.001^b$</td>
<td>1.10</td>
<td>1.93</td>
<td>1.93</td>
<td>1.93</td>
</tr>
<tr>
<td></td>
<td>Aspartate</td>
<td>$1.35 \times 10^{-20}$</td>
<td>$0.001^b$</td>
<td>1.45</td>
<td>3.08</td>
<td>0.72</td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td>C₃-P</td>
<td>$0.22 \times 10^{-20}$</td>
<td>$0.001^b$</td>
<td>0.25</td>
<td>0.53</td>
<td>0.53</td>
<td>0.53</td>
</tr>
<tr>
<td></td>
<td>Sucrose</td>
<td>$0.03 \times 10^{-20}$</td>
<td>$0.001^b$</td>
<td>0.05</td>
<td>0.13</td>
<td>0.02</td>
<td>0.003</td>
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<tr>
<td>PEP-CK</td>
<td>Malate</td>
<td>$1.83 \times 10^{-20}$</td>
<td></td>
<td>6.99</td>
<td>12.48</td>
<td>3.33</td>
<td>0.40</td>
</tr>
<tr>
<td></td>
<td>Pyruvate</td>
<td>$1.83 \times 10^{-20}$</td>
<td></td>
<td>6.76</td>
<td>11.85</td>
<td>11.85</td>
<td>11.85</td>
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<tr>
<td></td>
<td>Aspartate</td>
<td>$1.83 \times 10^{-20}$</td>
<td></td>
<td>8.88</td>
<td>18.89</td>
<td>4.38</td>
<td>0.52</td>
</tr>
<tr>
<td></td>
<td>PEP</td>
<td>$1.83 \times 10^{-20}$</td>
<td></td>
<td>9.01</td>
<td>19.17</td>
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<td>19.17</td>
</tr>
<tr>
<td></td>
<td>C₃-P</td>
<td>$0.61 \times 10^{-20}$</td>
<td></td>
<td>3.00</td>
<td>6.38</td>
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<td>6.38</td>
</tr>
<tr>
<td></td>
<td>Sucrose</td>
<td>$0.09 \times 10^{-20}$</td>
<td></td>
<td>0.56</td>
<td>1.47</td>
<td>0.30</td>
<td>0.03</td>
</tr>
</tbody>
</table>

$a$ Data from Hatch and Osmond (1976).

$b$ Values based on Weiner et al. (1988).
increasing the Stokes’ radius of a molecule; the specificity of diffusion inside micropores cannot be disregarded. Therefore, one must be aware that taking these processes into consideration will result in further increase of the concentration differences required to sustain diffusion.

Clearly C₄ plants do transport a large amount of photosynthates. Photosynthesis in C₄ plants, which might even exceed 40 μmol CO₂ m⁻² s⁻¹, produces a significant amount of assimilates exchanged between KMS and BS cells symplasmically. Thus: (i) other diffusion pathways apart from the plasmodesmal microchannels are involved; and (ii) another transport mechanism is involved in metabolic exchange between KMS and BS cells. These possibilities are considered below.

**Simple diffusion model: combined two-way metabolite exchange utilizing desmotubule and microchannels of plasmodesmata**

If combined two-way metabolic exchange is assumed, then the second route remains to be found. This would result in spatial separation of the transport from KMS to BS cells from that of the flux in the opposite direction. The desmotubule seems to be an ideal candidate. The role of desmotubules as a transport pathway was postulated many years ago, also in C₄ plants (Evert et al., 1977). Recently, this idea has been restated (Waigmann et al., 1997; Cantrill et al., 1999). One should underline, however, that there are strong arguments for the opinion that the desmotubule is a static, appressed structure at the centre of PD, not available for transport processes and acting as a structural component, often referred to as a central rod (Gunning and Overall, 1983; Tilney et al., 1991; Botha et al., 1993, Overall and Blackmann, 1996; Ding, 1998).

In this approach, it is assumed that metabolites moving from the KMS to the BS cells are transported inside desmotubules, while photosynthetic intermediates move from BS to KMS in plasmodesmal microchannels. Various possible desmotubule sizes (15, 25, and 35 nm in diameter) have been considered, with the resulting confinement factors (see Table 4) taken into consideration. The metabolite fluxes and the desmotubule diffusion coefficients were calculated as described in the section on ‘Metabolite fluxes and concentration differences required to sustain diffusion between KMS and BS cells’ and the concentration differences between photosynthetic cells, necessary to maintain the current net photosynthesis rates, were estimated (Table 5).

With the desmotubule assumed to be the additional transport pathway for diffusion, the required concentration differences between KMS and BS cells decreased significantly (Table 5). For NAD-ME species, when the widest desmotubule was taken into account, the differences were similar to the metabolite concentrations estimated experimentally. For other C₄ sub-types, these values were higher, but the difference was reduced to one order of magnitude only.

Participation of desmotubules in cell-to-cell transport was postulated by Waigmann et al. (1997) for cotton extrafloral nectary trichomes expelling large amounts of nectar. Desmotubules have also been postulated as a transport route in the symplasmic phloem loading mechanism (Gamalei et al., 1994). This sort of phloem-loading mechanism is related to so-called open (type 1) vein ultrastructure (Gamalei, 1991), where companion cells are connected to adjoining mesophyll cells by numerous plasmodesmata (more than 10 PD per μm² of the cell interface). Symplasmic phloem loading was postulated to be powered by polymer trapping mechanism (Turgeon, 1996), however, even in plants showing abundant plasmodesmata linking companion cells and mesophyll cells, for the transport of carbohydrates from photosynthetic cells to companion cells/sieve tube complex other mechanisms have been postulated as mass flow (Voitsekhovskaja et al., 2006) or even apoplastic transport (Turgeon and Medville, 2004).

In C₄ plants, metabolite concentration differences between KMS and BS cells obtained when desmotubular transport was assumed to occur were more realistic than those from other approximations. Thus, desmotubule involvement as a transport pathway in C₄ photosynthesis seems reasonable. However, it has been assumed that this pathway is available only for metabolites moving in one direction (i.e. from KMS to BS cells). Transport in the opposite direction remains a problem as there are only the microchannels available, and these require high values of δc (Table 5). This implies the involvement of transport mechanisms other than simple diffusion.

**Alternative mechanisms**

Apoplastic transport is an alternative to symplasmic transport. However, in the case of exchange of metabolites between KMS and BS cells, apoplastic transport may be questioned for two reasons. One is the suberin lamella within the KMS/BS walls of many C₄ plants, which nearly precludes apoplastic transport of solutes (Hattersley, 1987; Hattersley and Browning, 1981). It has also been shown that PCMBS, an inhibitor of the proton pump, has no distinct effect on photosynthesis in maize, a C₄ plant (Bourquin et al., 1990; Sowiński 1998), which clearly demonstrates that apoplastic transport is not involved in the photosynthetic transport in that species. Unfortunately, such studies have not been performed with other C₄ plants.

There are two possible alternatives to simple diffusion, the first being mass flow, postulated as an efficient means of cell-to-cell transport (Anisimov and Egorov, 2002; Voitsekhovskaja et al., 2006), and the second being
vesicular transport (Bil’ et al., 1976; Karpilov et al., 1976; Evert et al., 1977), similar to that postulated for proteins and other high molecular weight molecules and viruses (Chen and Kim, 2006). Vesicles could accumulate solutes to very high concentrations, using transporters located in the vesicle membrane. Vesicles could be unloaded at the plasmodesma neck region in a manner similar to the vesicle-mediated secreting transport system involving the vacuole and plasmalemma (Echeveria, 2000). The idea of a vacuole–desmotubule–vacuole continuum (Gamalei, 1996; Rinne et al., 2001) is intriguing in this context. However, we await experimental data to support these mechanisms.

Conclusions
In light of current knowledge on plasmodesma ultrastructure, the conventional model of C₄ photosynthetic intermediate exchange between KMS and BS cells based only on simple diffusion is not satisfactory, since the concentration differences for photosynthetic intermediates needed for cell-to-cell exchange of photosynthetic intermediates in KMS and BS cells seem unrealistic.

Theoretically, participation of desmotubules could improve transport efficiency in C₄ grasses, however, only in one direction (e.g. from KMS to BS cells). Transport in the opposite direction remains a problem as there are only the microchannels available, and these require high values of $\frac{\partial c}{\partial t}$ to enable a simple diffusion model to hold.

A more effective mechanism than simple diffusion is needed for cell-to-cell exchange of photosynthetic intermediates in C₄ plants. Two plausible mechanisms have been proposed here, but there is no experimental evidence to confirm that either of them is operative in C₄ plants.

Appendix
$c(i)$ concentration of metabolite $i$ (mol dm$^{-3}$)
$C_{BS}$ circumference of BS cells (μm)
$D(i)$ diffusion coefficient for metabolite $i$ in water (m² s$^{-1}$)
$D_{cyt}(i)$ diffusion coefficient for metabolite $i$ in cytoplasm (m² s$^{-1}$)
$D_{pmd}(i)$ plasmodesmatal diffusion coefficient for metabolite $i$ (m² s$^{-1}$)
$f_{PD}$ plasmodesmatal frequency per 1 mm² (mm$^{-2}$)
$\varphi$ surface fraction of plasmodesmatal microchannels in leaf segment of 1 mm²
$I_{BS}$ contribution of intercellular spaces to BS circumference
$IVD$ interveinal distance (μm)
$J(i)$ flux of metabolite $i$ (mol nm$^{-2}$ s$^{-1}$)
$k$ Boltzmann’s constant: $1.38 \times 10^{-23}$ J K$^{-1}$
$K_c$ confinement factor inside plasmodesmatal microchannel
$n_p(i)$ number of moles of metabolite $i$ transported between KMS and BS cells (mol)
$n_{ve}$ number of veins in a given volume of the leaf
$\eta$ viscosity of water: 1 mPa s
$r_K$ radius of plasmodesmatal microchannel: 2 nm
$r_{St}(i)$ Stokes’ radius for metabolite $i$ (nm)
$S_K$ cross-sectional area of single microchannel (nm²)

$S_W$ total area of KMS/BS cell walls in leaf segment of 1 mm² (nm²)
$T$ temperature (K)
$T_{PD}$ total area of plasmodesmatal microchannels cross-section in KMS/BS cell walls in leaf segment of 1 mm² (nm²)
$x$ diffusion pathway [nm]

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References


