Rapid report

Tree stem phospho*enol*pyruvate carboxylase (PEPC): lack of biochemical and localization evidence for a C_4-like photosynthesis system

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Received: *10 August 2007*
Accepted: *20 September 2007*

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Key words: beech (*Fagus sylvatica*), immunogold labelling, phospho*enol*pyruvate carboxylase (PEPC), ribulose-1,5-*bis*phosphate carboxylase/oxygenase (Rubisco), stem photosynthesis.

Summary

• Here, the kinetic properties and immunolocalization of phospho*enol*pyruvate carboxylase (PEPC) and ribulose-1,5-*bis*phosphate carboxylase/oxygenase (Rubisco) in young stems of *Fagus sylvatica* were investigated. The aim of the study was to test the hypothesis that there is a C_4-like photosynthesis system in the stems of this C_3 tree species.

• The activity, optimal pH and L-malate sensitivity of PEPC, and the Michaelis-Menten constant (K_m) for phospho*enol*pyruvate (PEP), were measured in protein extracts from current-year stems and leaves. A gel blot experiment and immunolocalization studies were performed to examine the isozyme complexity of PEPC and the tissue distribution of PEPC and Rubisco in stems.

• Leaf and stem PEPCs exhibited similar, classical values characteristic of C_3 PEPCs, with an optimal pH of *c*. 7.8, a K_m for PEP of *c*. 0.3 mM and a IC_{50} for L-malate (the L-malate concentration that inhibits 50% of PEPC activity at the K_m for PEP) of *c*. 0.1 mM. Western blot analysis showed the presence of two PEPC subunits (molecular mass *c*. 110 kDa) both in leaves and in stems. Immunogold labelling did not reveal any differential localization of PEPC and Rubisco, neither between nor inside cells.

• This study suggests that C_4-type photosynthesis does not occur in stems of *F. sylvatica* and underlines the importance of PEPC in nonphotosynthetic carbon fixation by most stem tissues (fixation of respired CO_2 and fixation via the anaplerotic pathway).

New Phytologist (2007) **176**: 775–781

doi: 10.1111/j.1469-8137.2007.02283.x

Introduction

In forest ecosystems, carbon (C) fluxes include plant respiration and photosynthesis. Although leaf photosynthesis represents the major part of the C assimilated by the plant, it is known that flowers, fruits and stems are able to photosynthesize (Aschan & Pfanz, 2003, 2006; Aschan *et al.*, 2005). We showed that current-year stems of European beech (*Fagus sylvatica*) are able to assimilate the equivalent of 40% of the C lost by respiration over a whole season (Damesin, 2003). Stem photosynthesis has been shown to be active in various tree species, with some of them exhibiting an instantaneous positive net CO_2 assimilation (Berveiller *et al.*, 2007).

The major source of assimilated C in stems is thought to be respiration (Nilsen, 1995; Cernusak & Marshall, 2000; Pfanz *et al.*, 2002; Wittmann *et al.*, 2005, 2006). Because of the high CO_2 concentration inside stems and their low permeability to gaseous diffusion (Pfanz *et al.*, 2002), very little external CO_2 reaches the chlorenchyma. CO_2 fixation in stems certainly occurs through ribulose-1,5-*bis*phosphate carboxylase/oxygenase (Rubisco) in the Calvin–Benson cycle. Rubisco activity was detected for the first time in bark tissues of *Populus tremuloides* by Schaedle & Brayman (1986), and we recently showed that young beech stems exhibit Rubisco activity, which remains nearly constant over the whole season (except just after budburst, when it is highest) (Berveiller & Damesin, in press). However, it has been proposed that phospho*enol*pyruvate carboxylase (PEPC; EC 4.1.1.31) can play a role in the regulation of CO_2 concentration and cytosolic pH by capturing part of the CO_2 (in its hydrated form) respired by stems. Höll (1973, 1974) was the first to report PEPC activity in wood of *Robinia pseudoacacia*. More recently, PEPC was detected in the bark chlorenchyma of *Pinus sylvestris* (Ivanov *et al.*, 2006) and in the whole stems of various tree species (Berveiller & Damesin, in press).

Some reports have recently proposed the possibility of a C_4-type pathway occurring in tree stems (Damesin, 2003; Ivanov *et al.*, 2006; Wittmann *et al.*, 2006; Berveiller & Damesin, in press). This concept is based on the paper by Hibberd & Quick (2002), which showed that photosynthetic cells of tobacco (*Nicotiana tabacum*) stems and petioles surrounding xylem and phloem possess high activities of enzymes characteristic of C_4 photosynthesis, especially the decarboxylating enzymes. These enzymes (NAD- and NADP-ME) allow the rapid decarboxylation of four-carbon organic acids, for example malate, fed from the xylem and phloem, thus releasing CO_2 for photosynthesis. This system suggests that this C_4 metabolite is synthesized via root PEPC activity, thus fulfilling the criterion of spatial separation of carboxylation activities (e.g. PEPC and Rubisco) characteristic of C_4 plants. If such tissues surrounding the vessels have in tree stems the same enzymatic equipment as in tobacco stems, a similar C_4-like photosynthesis system may be present. However, tree stems have relatively high PEPC:Rubisco activity ratios (10 for a C_4-plant leaf, 1 for a C_3-plant leaf, and 3 in tree stems; Berveiller & Damesin, in press) such that the classical C_4 photosynthesis system may also operate in the concentrically organized tissues of tree stems. This suggests a partial, or total, separation of PEPC- from Rubisco-containing cells. To the best of our knowledge, trees do not have a C_4 PEPC-encoding gene, but such a system may use a C_3-type PEPC, provided that its activity is high enough to sustain the C flux required by the pathway. In the present work, these questions have been addressed by investigating the biochemical properties and localization of PEPC (with respect to those of Rubisco) in stems of *Fagus sylvatica*.

Materials and Methods

Plant material

Samples of leaves and current-year stems of *Fagus sylvatica* L. (Fig. 1a) were obtained at the end of April 2006 from three adult trees at the University campus (University Paris-Sud, Orsay, France). Sampling was performed (at midday on the light-exposed part of the trees from the outer crown, at a height of *c.* 2–4 m, with a pruning hook) a few days after budburst; at this stage, young stems contained maximal amounts of soluble protein and showed high PEPC activity (Berveiller & Damesin, in press). Except in the case of chemical fixation, all the samples were immediately immersed in liquid nitrogen and stored at –80°C until use.

Protein extraction and enzyme assays

Frozen material (300 mg) was powdered under liquid nitrogen and ground in a buffer, as described in Gousset-Dupont *et al.* (2005). The addition of 3% (weight/weight (w/w)) polyethylene glycol (PEG) 4000 to the extraction medium was found to be required for maximal recovery of PEPC activity. Enzyme assays and the determination of the kinetic properties of PEPC were performed as described in Echevarria *et al.* (1994). The substrate (PEP) and L-malate response curves were determined at optimal (7.8) and suboptimal pH (7.3), respectively, to allow calculation of the Michaelis-Menten constant (K_m) for PEP (PEP concentration for 50% of maximal PEPC activity) and the IC_{50} for L-malate (the L-malate concentration that inhibits 50% of PEPC activity at the K_m for PEP). Total soluble protein contents were determined by the technique of Bradford (1976), with bovine serum albumin (BSA) as a standard.

Gel blot experiment

Aliquots (50 μg of proteins) of leaf and stem extracts were subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE; 8% acrylamide) (Laemmli, 1970) and immunoblot experiments were performed as described in Giglioli-Guivarc'h *et al.* (1996) using anti-C- and anti-N-terminal peptide antibodies and a chemiluminescent assay

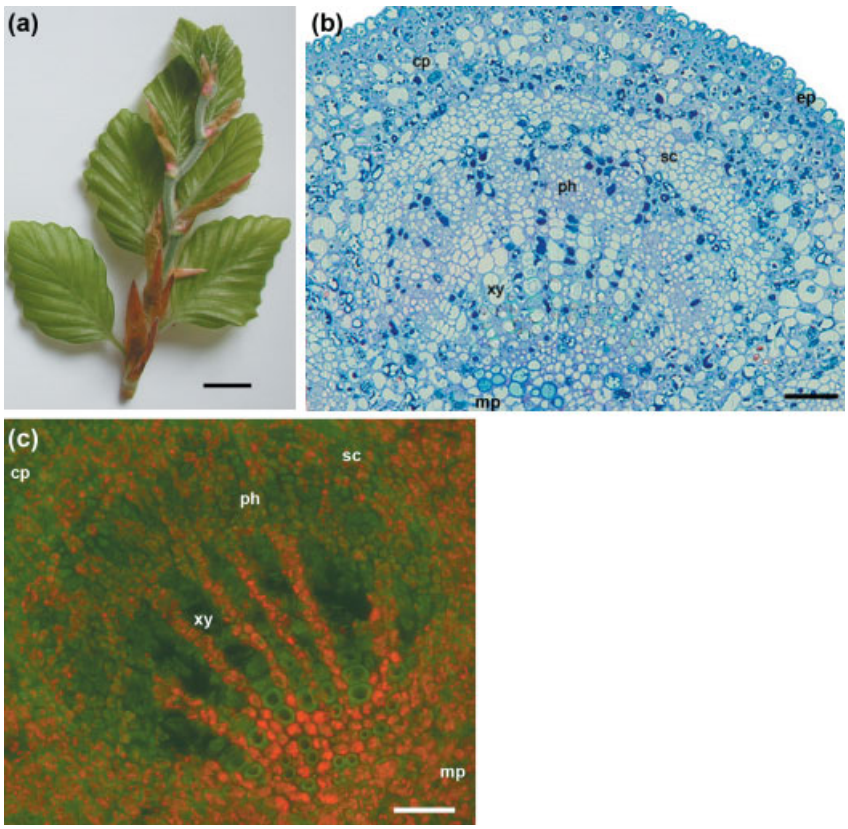

Fig. 1 Proxy photograph of a *Fagus sylvatica* leafy stem sampled at the end of April (a), and a semithin section of stem observed under a brightfield (b) and an epifluorescence (c) microscope. Bar: (a) 1 cm; (b, c) 50 μm. cp, cortical parenchyma; ep, epidermis; mp, medullary parenchyma; ph, phloem; sc, sclerenchyma; xy, xylem.

(Supersignal West Dura Signal; Pierce, Rockford, IL, USA) according to the manufacturer's instructions.

Sample preparation for light and electron microscopy

For epifluorescence observations, a transverse section (0.5 mm thick) of current-year stem was observed under an epifluorescence microscope (Axiophot; Zeiss, Rödermark, Germany) equipped with a blue excitation filter (BP 450–490) and an emission filter (LT 520). For brightfield and ultrastructure observations, small pieces of middle and apical portions of the stems were fixed in 3% glutaraldehyde and 1% paraformaldehyde in 100 mM cacodylate buffer (pH 7.3) in the presence of 1% (weight/volume (w/v)) tannic acid, 2% (w/v) sucrose, 0.1% Brij-35, and 2 mM $CaCl_2$ at room temperature for 6 h. It was necessary to place the mixture (fixer plus samples) in a vacuum for a few minutes (three times) to thoroughly impregnate the samples with the fixer. The samples were then washed with the cacodylate buffer and postfixed in 2% OsO_4 in cacodylate buffer for 1.5 h. They were then dehydrated though an ethanol series (10–95%) and embedded in Spurr's resin (Spurr, 1969). Transverse semithin sections (700 nm thick) of stem pieces were mounted on slides, stained with solution (Azure II 1% in water + methylene blue 1% in 1% borax) and observed under a standard upright microscope (BX 40; Olympus, Tokyo, Japan). For electron microscopy, transverse ultrathin sections (60 nm thick) of stem pieces were mounted on copper grids coated with Formvar (Monsanto Co., St Louis, MO, USA; polyvinylformal) and C, stained in uranyl acetate and lead citrate, and viewed under a transmission electron microscope (EM 300; Philips, Amsterdam, the Netherlands).

Immunogold labelling

Small stem samples were fixed in 0.5% glutaraldehyde and 4% paraformaldehyde (in the same buffer described above), dehydrated through an ethanol series and embedded in LRWhite resin (London Resin Co., Reading, UK). Ultrathin sections (60 nm thick) were collected on nickel grids coated with Formvar and C. The antisera used for immunogold labelling were anti-sorghum leaf C_4 PEPC antiserum and anti-maize leaf Rubisco large-subunit (LS) antiserum. They were used at a dilution of 1 : 1000 for Rubisco and 1 : 2000 for PEPC. The sections were stained with uranyl acetate. Some grids were coated with a preimmune serum as a control. The density of labelling was determined by counting the gold particles on several electron micrographs ($8 \leq n \leq 15$) and by calculating the number per unit area (μm^2).

Results and Discussion

Anatomy of young current-year stems

A few days after budburst, young stems of European beech were found to be wholly green (Fig. 1a) and phloem, xylem

Table 1 Kinetic and regulatory properties of phospho*enol*pyruvate carboxylase (PEPC) from leaves and current-year stems of *Fagus sylvatica*

	Optimal pH	PEPC activity at optimal pH (nmol min^{-1} mg^{-1} protein)	PEPC activity at pH 7.3 (nmol min^{-1} mg^{-1} protein)	PEP K_m (mM)	L-malate IC_{50} (mM)
Leaves	7.8	89.7 ± 4.4 (3)	48.5 ± 5.0 (6)	0.25 ± 0.08 (5)	0.07 ± 0.04 (4)
Stems	7.7	124.5 ± 15.4 (4) ns	62.6 ± 4.1 (5) ns	0.29 ± 0.07 (5) ns	0.11 ± 0.06 (4) ns

Values are mean ± SD (*n*). Data for stems and leaves were compared using a Student's *t*-test ($P = 0.05$).
PEP K_m, Michaelis-Menten constant, PEP concentration for 50% of maximal PEPC activity; L-malate IC_{50}, the L-malate concentration that inhibits 50% of PEPC activity at the K_m for PEP; ns, not significant.

and sclerenchyma were well developed (Fig. 1b). At this growth stage, the epidermis consisted of a single cell layer and the stem exhibited chlorophyll (Fig. 1c) and chloroplasts (Fig. 2a,b,c) in all cell types. In both cortical and medullar parenchyma, numerous chloroplasts could be seen, in contrast to the xylem, which contained numerous vessels (i.e. dead cells). These results are in good agreement with previous observations on the distribution of chlorophyll in the stem by epifluorescence microscopy (Dima *et al.*, 2006; Levizou & Manetas, 2006; Pilarski & Tokarz, 2006; Wittmann *et al.*, 2006; Berveiller *et al.*, 2007; Berveiller & Damesin, in press). In all tissues, the chloroplasts contained few grana stacks, which is a characteristic feature of light-adapted chloroplasts (Boardman, 1977). In contrast to our observations on buds and older stems (data not shown), there was no apparent gradient of grana stacks from the epidermis to the medullar parenchyma. On the basis of the presence of grana stacks and large starch grains, especially in parenchyma cells (Fig. 2a,c), the chloroplasts seemed to be photosynthetically functional.

Stem PEPC characteristics

We determined some biochemical properties of stem and leaf PEPCs. The leaf enzyme is a typical C_3-type PEPC known to be involved in the replenishment of tricarboxylic acid (TCA) cycle intermediates to sustain amino acid synthesis via the anaplerotic pathway (Latzko & Kelly, 1983; Lepiniec *et al.*, 1994; Stitt *et al.*, 2002).

SDS-PAGE analysis (Fig. 3a) showed very similar protein profiles for leaves and current-year stems, with the large subunit of Rubisco (*c.* 55 kDa) being the quantitatively predominant protein of the extracts. It was also noted that Rubisco was less abundant in stems than in leaves, in accordance with Rubisco activity measurements (Berveiller & Damesin, in press). Western blot analysis using C- and N-terminal PEPC antibodies as probes (Fig. 2b) revealed two PEPC bands with similar intensities (*c.* 110-kDa), which should correspond to different isoforms (as the subunits are intact proteins). Indeed, C_3 plants possess several genes encoding PEPC (classically four) which are differently expressed according to the organ (Svensson *et al.*, 2003; Gousset-Dupont *et al.*, 2005; Lebouteiller *et al.*, 2007).

The optimal pH for the enzyme was 7.8 and 7.7 in leaf and current-year stem extracts, respectively (Table 1). On a protein basis, the PEPC activity was 1.3-fold higher in current-year stems than in leaves (the difference was nonsignificant), when measured at both the optimal pH and at pH 7.3. The activity ratio (pH 7.8 : 7.3) generally reflects the phosphorylation state of the enzyme; that is, the ratio decreases when the phosphorylation state increases (Echevarria *et al.*, 1994). It was found to be 1.9 and 2.0 for leaves and stems, respectively, suggesting that the enzyme has a similar phosphorylation state in the two organs (at the stage of harvest).

Substrate response curves (PEP) were hyperbolic in shape, with similar K_m values of 0.25 and 0.29 mM for leaves and stems, respectively (Table 1), which are close to values characteristic of a C_3-type PEPC; C_4-type enzymes have K_m values of *c.* 2.5 mM (O'leary, 1982). The L-malate IC_{50} values were also typical of C_3 plant PEPCs and 10-fold lower than those displayed by C_4 plant PEPCs (O'leary, 1982). Taken together, these results show that the current-year stem PEPC exhibits the typical kinetic properties of a C_3 plant enzyme, with no large increase in content (as compared with leaves). This is not indicative of the occurrence of a C_4-like system in this organ.

Localization of Rubisco and PEPC in stem tissues

C_4 plants are classically characterized by a spatial separation of the photosynthetic pathway in two structurally and biochemically specialized photosynthetic cell types (Kranz anatomy). In addition, recent results have shown that C_4-like photosynthesis may occur in single cells of C_3 plants, for example *Hydrilla verticillata* (Magnin *et al.*, 1997) and *Borszczsowia aralocaspica* (Voznesenskaya *et al.*, 2001); in *H. verticillata*, an aquatic plant, the C_4 system is inducible, while in *B. aralocaspica* there occurs a polarity in specialized chloroplasts. In stems of *F. sylvatica*, we have reported a relatively high PEPC:Rubisco activity ratio (Berveiller & Damesin, in press). This prompted us to examine whether the two carboxylases are totally or partially separated in the concentrically organized cells of tree stems, such that a C_4-like photosynthesis system could be operative in this organ, in addition to the system proposed by Hibberd & Quick (2002).

In current-year stems of *F. sylvatica*, Rubisco and PEPC were detected in all investigated tissues (Fig. 3d–i). Until now, the only study considering this aspect of tree stem photosynthesis was that by Buns *et al.* (1993). These authors detected

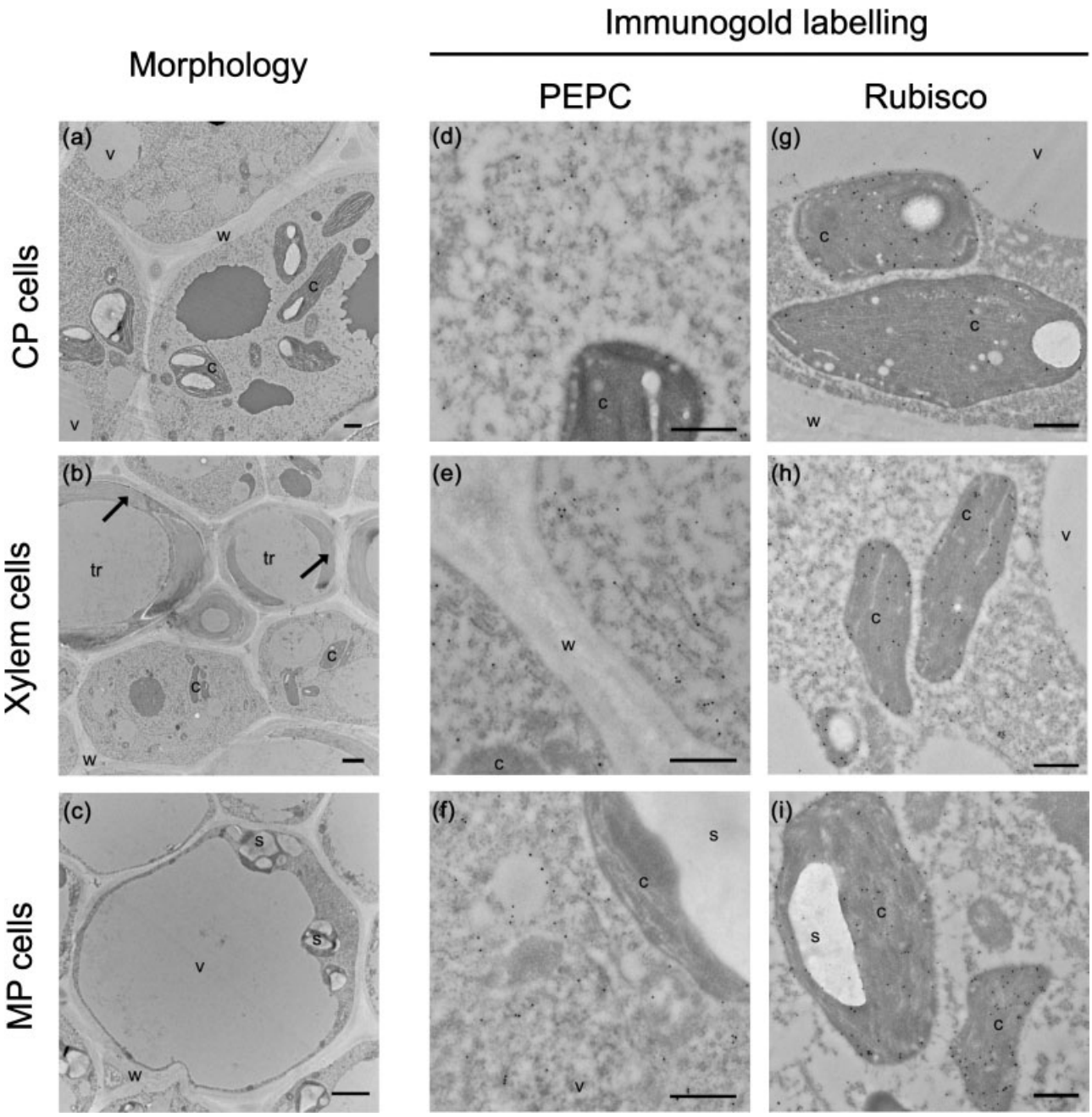

Fig. 2 Ultrastructural anatomy of current-year stems of *Fagus sylvatica* (a, b, c) and immunogold labelling of phospho*enol*pyruvate carboxylase (PEPC) (d, e, f) and the Rubisco large subunit (g, h, i) in these stems. (b) Arrows indicate the secondary wall in the process of formation during tracheid development. c, chloroplast; CP, cortical parenchyma; MP, medullary parenchyma; Rubisco, ribulose-1,5-*bis*phosphate carboxylase/oxygenase; s, starch; tr, tracheid; v, vacuole; w, wall. Bars: (a, c) 2 μm; (b) 5 μm; (d–i) 0.5 μm.

a low density of Rubisco labelling in twigs of *Taxus baccata* in comparison to needles. Similarly, our results showed a low density of Rubisco and PEPC labelling (Table 2). The high labelling detected in the cytosol in the case of PEPC (a cytosolic enzyme), and in chloroplasts in the case of Rubisco LS, together with a low background (statistically different from PEPC and Rubisco labelling and close to that of the pre-immune serum; data not shown), showed that the technique is valid and particularly well adapted to this material. It appeared that the chloroplasts accumulated significantly more Rubisco (approximately twice as much) in xylem cells than in cortical or medullar parenchyma cells. This observation supports the Hibberd and Quick hypothesis as it suggests a functional link between these cells and xylem vessels. The vessels provide CO_2 (probably via malate and corresponding decarboxylating enzymes) to xylem cells, thereby elevating its concentration in

Table 2 Immunogold labelling of phospho*enol*pyruvate carboxylase (PEPC) and ribulose-1,5-*bis*phosphate carboxylase/oxygenase (Rubisco) in the different cell types of current-year stems of *Fagus sylvatica*

		Number of gold particles per μm²		
	Cell fraction	CP cells	Xylem cells	MP cells
PEPC	Cytosol	7.2 ± 0.7 (14)[a]	7.1 ± 0.6 (13)[a]	6.4 ± 0.4 (8)[a]
	Organelles	2.4 ± 0.6 (14)	1.3 ± 0.2 (13)	1.0 ± 0.2 (8)
Rubisco LS	Chloroplasts (starch grains omitted)	16.8 ± 2.4 (10)[a]	31.5 ± 3.1 (15)[b]	19.6 ± 1.5 (14)[a]
	Cytosol + other organelles	3.5 ± 0.5 (10)	6.0 ± 1.3 (15)	3.5 ± 0.4 (14)

The number of gold particles per μm² is given as mean ± SD (*n*). Within the same row, a different superscript letter indicates a significant difference at $P < 0.05$ (Student's *t*-test).
CP, cortical parenchyma; MP, medullary parenchyma; Rubisco LS, Rubisco large subunit.

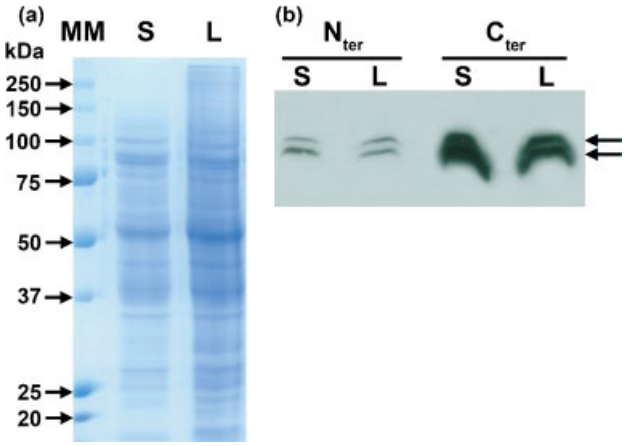

Fig. 3 Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE; 8% acrylamide) of total soluble protein (a), and western blot analysis of phospho*enol*pyruvate carboxylase (PEPC) with anti-N-terminal (N_{ter}) and anti-C-terminal (C_{ter}) antibodies (b) from leaves (L) and current-year stems (S) of *Fagus sylvatica*. (b) Arrows indicate the two PEPC isoforms. MM, molecular mass markers.

the vicinity of Rubisco. This hypothesis may be supported further by the presence of light in vascular tissues of tree stems (Sun *et al.*, 2003). However, the uniform localization of PEPC in all tissues, the absence of a preferential distribution of chloroplasts in cells, and the PEPC kinetic data suggest that a C_4-like system based on the separation of carboxylating enzymes, or on the single cell system (as described in *B. aralocaspica*), is not operative in stems. The current dogma is that Rubisco refixes CO_2 liberated by respiration in stems, thus making O_2 available for this process under light conditions. This is important in this organ to avoid anoxia and perturbation of wood formation (Pfanz *et al.*, 2002). In this context, PEPC should also actively contribute to CO_2 refixation in the light, but allows this process to be continued in the dark.

Acknowledgements

We gratefully acknowledge Jean-Louis Prioul for the gift of Rubisco LS antibodies. This research was financed by the IFR87 'La Plante et son Environnement' (Gif-sur-Yvette, CNRS, France) and the French projects 'Ministère délégué à la recherche – ACI Jeunes Chercheurs' no. JC10009 and 'Programme National ACI/FNS ECCO, PNBC' (convention no. 0429 FNS). The ESE laboratory is supported by the University of Paris-Sud, the Centre National de la Recherche Scientifique (CNRS), and AgroParisTech.

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