Plant, Cell and Environment (2006) **29**, 15–25

Effects of cold-girdling on flows in the transport phloem in *Ricinus communis:* is mass flow inhibited?

ANDREAS D. PEUKE[1,*], CAREL WINDT[2,*] & HENK VAN AS[2]

[1]*University of Freiburg, Chair of Tree Physiology, Georges-Köhler-Allee 53, D-79110 Freiburg i. Br., Germany and*
[2]*Wageningen University, Laboratory of Biophysics and Wageningen NMR Centre, Dreijenlaan 3, 6703 HA Wageningen, the Netherlands*

ABSTRACT

The effects of cold girdling of the transport phloem at the hypocotyl of *Ricinus communis* on solute and water transport were investigated. Effects on the chemical composition of saps of phloem and xylem as well as of stem tissue were studied by conventional techniques and the water flow in the phloem was investigated by NMR imaging. Cold girdling reduced the concentration of sucrose but not that of inorganic solutes or amino acids in phloem saps. The possibility that cold treatment inhibited the retrieval of sucrose into the phloem, following leaching from the sieve tubes along a chemical gradient is discussed. Leaching of other solutes did not occur, as a result of missing promoting gradients in stem tissue. Following 3 d of cold girdling, sugar concentration increased and starch was synthesized and accumulated in stem tissue above the cold girdling region and along the cold-treated phloem pathway due to leaching of sugars from the phloem. Only in the very first period of cold girdling (< 15–30 min) was mass flow inhibited, but recovered in the rest of cold treatment period to values similar to the control period before and the recovery period after the cold treatment. It is concluded that cold treatment affected phloem transport through two independent and reversible processes: (1) a permanent leaching of sucrose from the phloem stem without normal retrieval during cold treatment, and (2) a short-term inhibition of mass flow at the beginning of cold treatment, possibly involving P proteins. Possible further mechanisms for reversible inhibition of water flow are discussed.

Key-words: *Ricinus communis*; cold girdling; NMR-imaging; phloem; water flow.

INTRODUCTION

One of the most important features that have allowed higher plants to conquer dry land are the phloem and xylem which transport water, photo-assimilates, nutrients and signals over long distances within the plant. The phloem is responsible for the transport of metabolic products and for the recycling of mineral nutrients from the shoot to the root or within the shoot from mature leaves to younger growing parts. The phloem can be divided into three functional areas: loading, transport and unloading phloem (van Bel 1993, 2003a, b). While the loading phloem is responsible for active loading of photoassimilates into the phloem, the unloading phloem delivers assimilates to sink tissues, and the transport phloem translocates water and solutes from source to sink. In the transport phloem, which generally can be found from the main leaf veins downwards, release as well as loading (retrieval) of sugars takes place (Patrick 1997; van Bel 1998, 2003a, b; Komor 2000; Lalonde *et al*. 2003; Gould, Minchin & Thorpe 2004). Along the way the transport phloem provides the surrounding tissue with assimilates for processes such as maintenance and growth.

The forces that drive phloem transport and dictate the direction of flow originate in the source–sink relations between the phloem and the tissue surrounding it (van Bel 1998, 2003a, b; Bancal & Soltani 2002; Henton *et al*. 2002; Thompson & Holbrook 2003; Lalonde *et al*. 2003; Gould *et al*. 2004). It has been shown that in the phloem, but also in the xylem, complex and fragile gradients in pressure and osmotic potential exist that are easily disturbed by invasive experimentation (van Bel 1998, 2003a, b;Bancal & Soltani 2002; Lalonde *et al*. 2003). It is widely accepted that these potential gradients drive long-distance transport in the phloem as well as the xylem, even though the current debate around the validity of the cohesion tension theory (Zimmermann *et al*. 2004) has demonstrated that not everybody agrees on the question of how large the water potential gradients in xylem vessels can be.

Long-distance phloem transport within intact plant systems is especially difficult to measure because only few techniques are suitable and because of the extreme sensitivity of the phloem to wounding. In a number of studies dyes or radioactive tracers were applied or injected to investigate transport in the phloem (see *inter alia* Patrick 1997; Knoblauch & van Bel 1998; Oparka & Turgeon 1999; Komor 2000). Additionally, the small vessel sizes, the slow flow velocities and the small flowing volume per unit of

Correspondence: Andreas D. Peuke. Institut für Forstbotanik und Baumphysiologie, Professur für Baumphysiologie, Georges-Köhler-Allee Geb. 053/054, D-79110 Freiburg im Breisgau/ Germany. Fax: + (0)761 203 8302; e-mail: AD_Peuke@web.de

**These authors contributed equally to this work.*

cross-section make measuring phloem flow a technically challenging enterprise. Currently, the method that has been most successful in providing detailed, non-invasive information on the characteristics of water transport in the phloem of intact plants is nuclear magnetic resonance (NMR) flow imaging. The first (non-imaging) method to measure xylem water transport was presented about 20 years ago (Van As & Schaafsma 1984; Reinders, Van As & Schaafsma 1988; Schaafsma, Van As & Palstra 1992). In 1988, Jenner *et al*. performed flow imaging and observed *in vivo* water movement on a submillimetre scale in developing grains of wheat, by using a pulsed gradient spin echo nuclear magnetic resonance technique combined with micro imaging (Jenner *et al*. 1988). With flow-sensitive NMR imaging it was possible to measure xylem water flow in single vascular bundles of maize plants (Kuchenbrod *et al*. 1996). Using a similar flow imaging method, Köckenberger *et al*. (1997) were able to measure phloem water transport as well as xylem transport in very young, non-transpiring seedlings of *Ricinus*. NMR flow imaging can be used to visualize the location of xylem and phloem in a cross-section of the plant stem, as well as delivering detailed information regarding the characteristics of flowing water. The amount of stationary and flowing water per pixel, the linear velocity of the flowing water per pixel, and the total volume flow per pixel can be accurately quantified (Scheenen *et al*. 2000b, 2001). Rokitta, Zimmermann & Haase (1999) developed a rapid method to measure xylem and phloem mass flow within three to seven minutes, respectively, trading image resolution and the ability to record detailed flow profiles for the ability to measure quickly. Using the former method, Peuke *et al*. (2001) monitored the mass flow in both xylem and phloem during a daily light/dark cycle with a high time resolution. C.W. Windt, F.J. Vergeldt, P.A. de Jager & H. Van As (unpublished data) were the first to quantitatively measure detailed flow profiles (called propagators) of phloem flow, on large and fully developed plants which could be placed upright in a dedicated NMR imager, and grown and measured for extended periods of time.

Numerous papers have shown that the transport of assimilates in the phloem is inhibited by cold or ice treatment (Giaquinta & Geiger 1973; Minchin & Thorpe 1984; Lang & Minchin 1986; Grusak & Minchin 1989; Pickard & Minchin 1990; Hannah, Iqbal & Sanders 2001; Gould *et al*. 2004) as well as by the application of heat, vibration, electric or osmotic treatment. Cold treatment of the phloem was also used as a tool in plant physiology to change the distribution of photo-assimilates over the plant (Candolfi-Vasconcelos, Candolfi & Koblet 1994). In the cited papers the authors investigated the effect of different treatments on the transport of photo-assimilates in the phloem, and found responses described as an 'inhibition' (but not total stoppage) (Giaquinta & Geiger 1973; Grusak & Minchin 1989; Hannah *et al*. 2001), 'blockage' (Minchin & Thorpe 1984; Gould *et al*. 2004) or 'cessation' (Pickard & Minchin 1990) of phloem transport. However, because mainly only the transport of photo-assimilates was measured, it is not clear whether the treatments caused a total stoppage of water transport in the cold-treated phloem as well. The latter may not necessarily be the case.

The aim of the present paper was to investigate the effects of cold girdling on the sap flow in the transport phloem in *Ricinus*, and to distinguish between solute transport, by monitoring changes in solute concentrations, and water mass flow, by means of NMR flow imaging. For the first time the results of NMR flow imaging on phloem water transport in adult, well-transpiring plants under control as well as under cold girdling conditions are presented.

MATERIALS AND METHODS

Plant material

Seeds of *Ricinus communis* L. were germinated in vermiculite moistened with 0.5 mM $CaSO_4$. After 13–15 d the plants were transferred to substrate, one plant in a pot of 5 L. The substrate consisted of two parts commercial potting soil (Floradur®; Floragard GmbH, Oldenburg, Germany) and one part Perlite (Perligran® G; Deutsche Perlite GmbH, Dortmund, Germany). Every third day the pots were well watered with tap water and after the first month the plants were supplied every second week with a commercial fertilizer (0.3% Hakaphos® Blau; Compo GmbH, Münster, Germany).

The plants were cultivated in a greenhouse (15–25 °C; 45–70% relative humidity) with additional illumination during a 16 h photoperiod (Osram HQL 400, Osram GmbH, Münster, Germany; 300–500 μmol photon m^{-2} s^{-1}).

Cold-girdling

Two sets of short-term cold girdling experiments with a total duration of 8 h were performed, and one long-term set of experiments with a duration of 3 d. The first short-term cold girdling experiment was used to monitor the effects of cold girdling on phloem sap composition, the second to measure the effect of cold girdling on phloem water mass flow by means of NMR flow imaging. The long-term cold girdling experiment was used to study the effect of sustained cold girdling on the chemical composition of phloem sap, xylem sap and on the chemical composition of the tissue at different positions along the length of the stem.

In the first set of short-term experiment 20 mL plastic vials were fitted to the hypocotyl and sealed water tight with dental rubber material. The vials were filled with water at room temperature, which was replaced with ice water to start the cold treatment. The cold-girdling treatment was ended by removing the ice water, and subsequently replacing it with warm water at room temperature. The second set of short-term cold girdling experiment took place inside the NMR set-up, making it necessary to use an alternative method to apply a cold girdling treatment without touching or moving the plant. In order to do so, a heat exchange tube was wrapped around the hypocotyl over a length of approximately 5 cm and connected to a cryostat. Using this set-up

a temperature of around 0 °C, measured directly above the cold girdle, could be achieved within 5–15 min (speed of cooling: 1.3–5° min^{-1}). In the long-term experiment the same cold girdling set-up was used to impose the prolonged cold treatment. In all experiments the cold girdle was placed at the hypocotyl about 1 cm below the node of the cotyledons (Fig. 1a).

Collecting of sap samples and harvesting of plant material

Phloem sap was collected below the cold-girdling region (see Fig. 1a) according to Pate, Sharkey & Lewis (1974). Xylem sap was obtained as root pressure exudate at the time of harvesting axis material directly above the cold girdle. The first droplets of xylem or phloem sap that appeared upon cutting or incision were removed to avoid contamination with the content of cut cells or reverse flow. During short-term experiments, small volumes of phloem sap were collected below the cold girdle after shallow incisions into the cortex of the hypocotyls, with an interval of 15 min between samples. The collection of 3 μL of phloem sap lasted between 3 and 5 min. After long-term cold girdling 20 μL of phloem sap were collected by the same method. Afterwards the plants were decapitated above the cold girdle and xylem root pressure saps were harvested from the stump for about 20–30 min. Although solutes in root pressure xylem saps are higher concentrated than in the xylem sap of transpiring plants, the ratio of solutes in shortly collected root pressure saps is not affected. The axis of the plant at the hypocotyl was divided in sections of 4 ± 1 cm in the direction of the phloem flow basipetally from the shoot: (I) the second region above; (II) the first region above; (III) the region of the cold girdle; and (IV) the region below the cold girdle (see Fig. 1). The parts were washed and, after freezing and lyophilizing, the material was ground. Dried powder was used for chemical determinations.

Chemical analysis

Saps were analysed directly, without further extraction. Potassium in the phloem saps after short-term cold treatment was measured after dilution with an ionization buffer

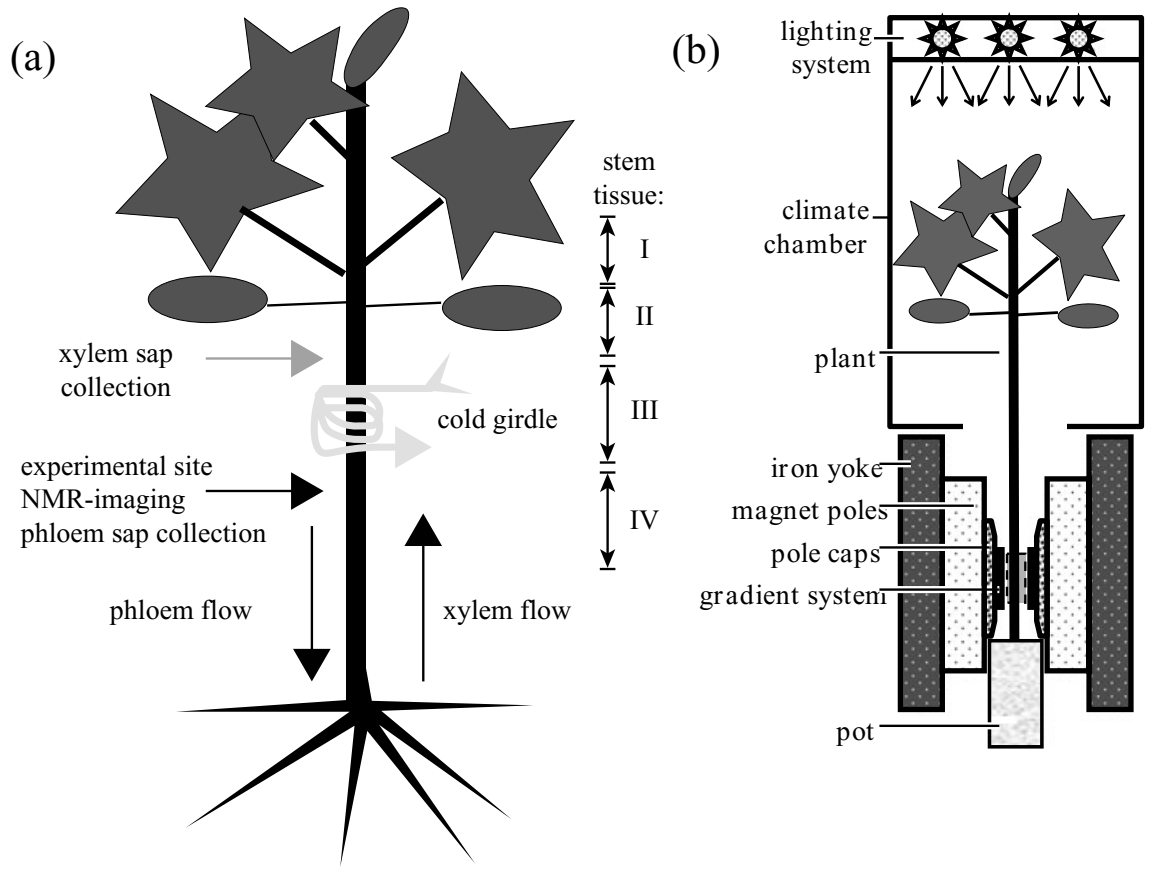

Figure 1. Experimental design. (a.) Cold girdling, phloem sap collection and NMR-imaging were done on the main stem/axis of the plants at different heights, as indicated. After long-term cold girdling four stem sections of the hypocotyl were harvested: the second section acropetally above cold girdle (I), the first section acropetally above cold girdle (II), the cold girdling section (III) and the next basipetally section below cold girdling (IV), regarding the phloem flow direction, respectively. Every section was approximately 4 ± 1 cm. (b) Schematic overview of the NMR imaging setup; the custom built RF coil, which is wrapped around the stem of the plant and positioned in the centre of the magnet, in between the gradient plates. During operation the coil is shielded from ambient RF radiation by means of aluminium foil.

(CsCl 9.4 mol m^{-3}, $Sr(NO_3)_2$ 57.2 mol m^{-3}) by atomic absorption spectrometry (FMD 3; Carl Zeiss, Oberkochen, Germany). Sucrose in the short-term phloem sap was measured by refractometry.

Carbon and nitrogen in the plant tissue was determined by use of a CHN-analyser (CHN-O-RAPID; Heraeus, Hanau, Germany). The elemental composition of the plant tissue was analysed using an ICP spectrometer (JY 70 plus; ISA, Instrument S.A. division Jobin-Yvon, France) after digestion with nitric acid under pressure for 10 h at 170 °C. For the determination of carbohydrates, dry matter was extracted with water and after centrifugation the pellet was incubated with amyloglucosidase. Soluble carbohydrates were estimated in the water extract and starch after enzymatic digestion by the method of Roe (1955).

In long-term experiments the elemental composition in saps was determined by ICP spectrometry (see above). Amino acids were determined using an amino acid analyser (Biotronik Co., Maintal, Germany). The amino acids were separated in this high-performance liquid chromatography system by ion exchange and detected after postcolumn derivatization with ninhydrin at 570 nm.

NMR imaging set-up

The MRI system was a Bruker Advance console (Bruker, Karlsruhe, Germany) in combination with a Bruker electromagnet (Bruker, Karlsruhe, Germany), generating a magnetic field strength of 0.72 T over a 10 cm air gap. The magnetic field was stabilized with an external ^{19}F lock unit (Bruker, Karlsruhe, Germany). A shielded gradient system with a planar geometry and maximum field strength of 1 T m^{-1} (*X*, *Y* and *Z* directions) was used (Resonance Instruments Ltd, Witney, UK). An air gap of 50 mm between the two gradient plates provided open access, from the front and back of the gradient set, as well as from above and below.

A solenoid radio frequency (RF) coil for induction and detection of the NMR signal was custom-made for each individual plant. First, a loosely fitting mould with a diameter of 10 mm was put around the stem of the plant. The RF coil was then constructed by wrapping nine turns of 0.5 mm copper wire around the mould. The finished coil was connected to a compact, home-built tuning circuit, electromagnetically shielded by means of aluminium foil and copper tape, and fixed to a rod next to the plant. The assembly of pot, plant and coil was then inserted, upright, into the magnet (Fig. 1b). The conditions in the magnet were 25 ± 1 °C at daytime and 22 ± 1 °C in the night, 20–27% RH and 200 μmol photon m^{-2} s^{-1}).

NMR flow imaging

Flow imaging was performed using the method described by C.W. Windt *et al.* (unpublished data), employing either a pulsed field gradient–spin echo–turbo spin echo (PFG-SE-TSE) sequence (Scheenen *et al.* 2000a), or a pulsed field gradient–stimulated echo–turbo spin echo (PFG-STE-TSE) sequence (Scheenen *et al.* 2001) when long flow labelling times were required for measuring very low flow velocities (phloem transport). In both cases linear displacement was measured by stepping the amplitude of the PFGs from $-G_{max}$ to $+G_{max}$ and sampling q-space completely. After Fourier transformation of the signal as a function of G, the complete distribution of displacements of water in the direction of G within the labelling time Δ (called propagator, see, e.g. Scheenen *et al.* 2001) was obtained for every pixel in an image. From these single pixel propagators, the following flow characteristics were extracted for each volume element in the image, as described by Scheenen *et al.* (2000b): the total amount of water (Fig. 2a & e), the amount of stationary water (Fig. 2b & f), the amount of flowing water (Fig. 2c & g), the average linear velocity (including the direction of flow), and the volume flow (Fig. 2d & h). A single flow measurement typically took between 15 and 30 min (for a phloem flow measurement with 32 G-steps and an image matrix of 64 by 64 pixels). Data analysis was performed in IDL (Research Systems Inc., Boulder, CO, USA), using home-built fitting and calculation routines.

In order to construct spatially resolved phloem flow maps (amount of flowing water per pixel, average linear velocity per pixel, and volume flow per pixel), the data of at least four individual flow measurements were averaged in order to improve the signal-to-noise (S/R) ratio, and then analysed on a per-pixel basis (Fig. 2b). In order to quantify phloem flow with a higher time resolution (one data point per individual flow measurement), the pixels containing phloem flow were identified as noted above. The propagators of these selected pixels were subsequently summed and the resulting one-dimensional total propagator (Fig. 3) analysed, to yield a quantitative flow profile of all flowing water within the phloem flow mask. Due to random diffusion, the stationary pool of water exhibited a velocity distribution that was symmetrical around zero. The spatial resolution of both xylem and phloem flow measurements was more than sufficient to separate the pixels that contained the signal from the rapidly upward moving xylem sap, from the pixels that contained the slower downward moving phloem sap at the hypocotyl of *Ricinus*. Furthermore, the flowing xylem and phloem water was detected exactly where it was expected on the basis of anatomy.

Phloem flow measurements were carried out using the following experimental parameters: image matrix 64 pixels by 64 pixels, field of view 9 mm by 9 mm, slice thickness 6 mm, spectral width (SW) 25 kHz, repetition time (TR) 1500 ms, four times averaging, first echo time (TE_1) 9.21 ms, all other echoes echo time (TE) 7.41 ms, turbo factor 8; flow encoding: labelling time Δ 400 ms, PFG duration δ 2.5 ms, 32 PFG steps, PFG_{max} 0.192 T m^{-1}; acquisition time 30 min. For xylem flow measurements the experimental parameters were set as follows: image matrix 128 × 128, TR 2500 ms, two times averaging, SW 50 kHz, FOV 9 mm × 9 mm, slice thickness 3 mm; flow encoding: Δ 16 ms, δ 2.5 ms, 32 PFG steps, PFG_{max} 0.461 T m^{-1}; acquisition time 22 min

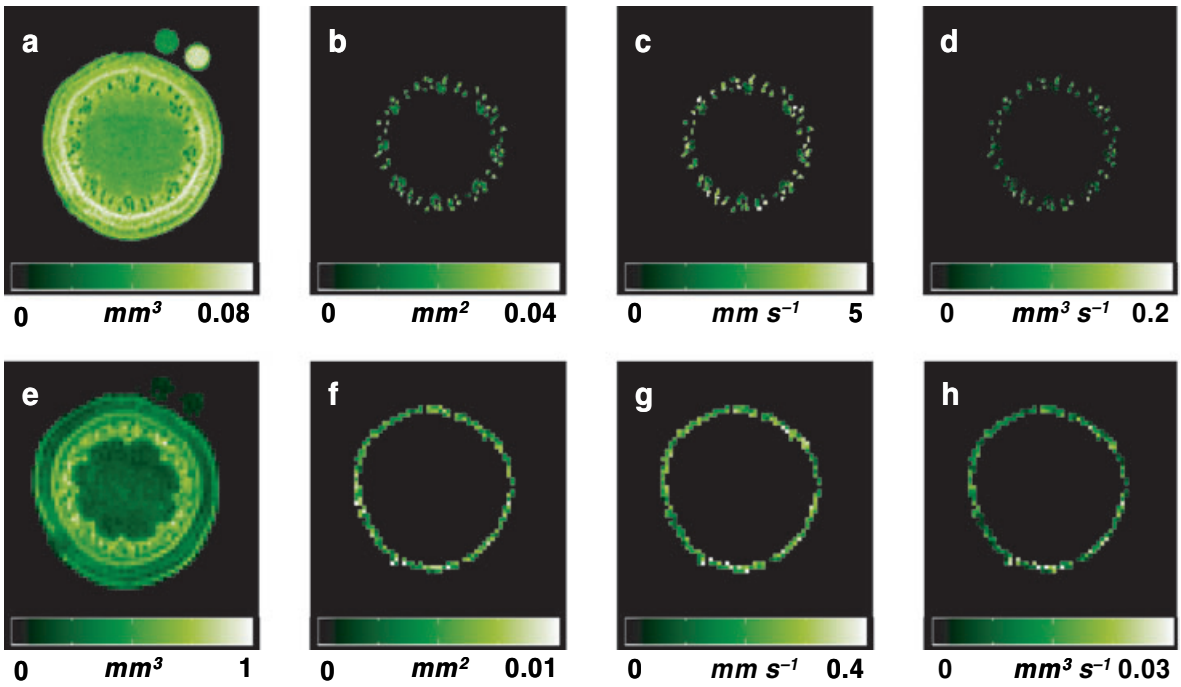

Figure 2. Quantitative NMR flow images of xylem water moving upwards through the hypocotyl of *Ricinus communis* (a–d), and phloem water moving downwards to the roots (e–h). Shown are the volume of stationary water per pixel (a and e), the volume of flowing water per pixel (b and f), the average linear velocity per pixel (c and g), and the average volume flow per pixel (d and h). The xylem flow images were calculated from a single flow imaging measurement; the phloem flow images were constructed from seven consecutive individual flow imaging measurements. The differences in the amounts of water per pixel shown in parts a and d are due to differences in image matrix size (128 × 128 versus 64 × 64), slice thickness (3 versus 6 mm) and scaling.

Statistics

All statistical calculations were performed with SAS release 8.2 (SAS Institute, Cary, NC, USA. One-way analyses of variance were performed by the GLM procedure.

RESULTS

Effects of short-term cold-girdling on phloem sap sucrose and potassium concentrations

During short-term cold-treatment experiments, small phloem sap samples were collected below the cold-girdle (see Fig. 1a). Samples were taken every 15 min in order to achieve a high time resolution. Due to the small sample volume only the two most concentrated solutes were determined: sucrose as the major organic and potassium as the major inorganic compound. During the cold treatment, the sucrose concentration in the phloem dropped to 88% of the value before cold girdling (418 ± 11 mM, Fig. 4). Upon rewarming of the cold girdling region, the sucrose concentration of the phloem sap recovered to a concentration comparable to the concentration before cold girdling (411 ± 13 mM). Although the effect of cold girdling on the sucrose concentration of the phloem sap was relatively small, the values during cold-treatment were significantly lower than before and after the cold treatment. In contrast to sucrose, the concentration of potassium in the phloem samples (56.6 ± 3.3 mM) was not affected by cold-girdling (moreover, potassium was not significantly changed in a daily time course in *Ricinus*, Peuke *et al*. 2001).

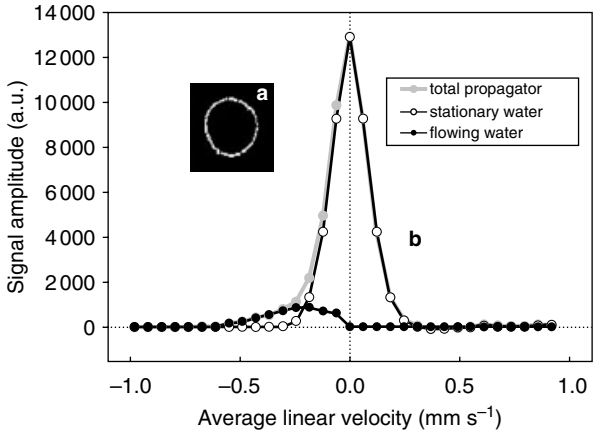

Figure 3. Flow profile (total propagator) of all pixels containing water flowing in a downward direction. The summation of all pixels in the phloem flow mask (a) yields a total propagator (b, grey line), which can be used to separate the signal arising from the freely diffusing stationary water (black line with white markers) from the signal originating from the flowing phloem water (black line with black markers).

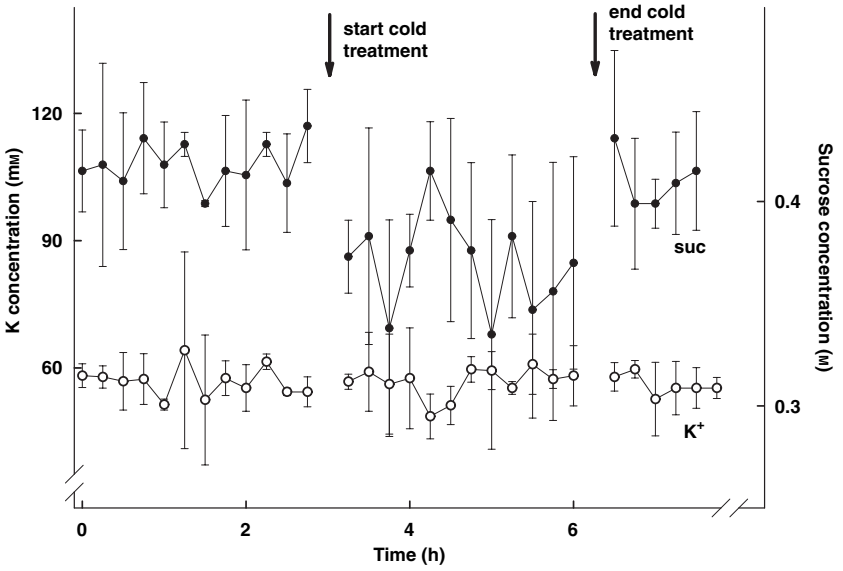

Figure 4. Sucrose and potassium concentration in phloem sap collected below cold-girdle at the hypocotyl of 45–55-day-old *Ricinus communis*, before and during cold treatment (arrow/'start cold treatment') and during the subsequent recovery phase (arrow/'end cold treatment').

Effects of long-term cold-girdling on the chemical composition of phloem sap, xylem sap and stem tissue

During the long-term cold girdling experiments, the concentrations of sucrose and potassium in the phloem sap changed in a fashion similar to the changes that took place during short-term cold girdling. After 3 d of cold treatment, the concentration of sucrose in the phloem sap was lowered significantly, from 436 ± 22 mM before cold girdling, to 353 ± 43 mM after (a decrease of 19%, Fig. 5). The concentration of potassium in the phloem sap was unaffected by the prolonged cold treatment (51.8 ± 2.1 mM, compared with 51.6 ± 2.9 mM before cold treatment). Similarly the concentrations of sodium (0.50 ± 0.03 mM), calcium (1.9 ± 0.3 mM), magnesium (2.6 ± 0.2 mM), phosphorus (10.7 ± 0.4 mM), sulphur (3.7 ± 0.2 mM), and amino acids (35.4 ± 3.7 mM) remained unchanged by the cold treatment.

In order to measure the effects of cold-girdling on the chemical composition of the transpiration stream, xylem exudate was collected after decapitating the plant directly above the cold-girdle (Fig. 1a). Long-term cold-girdling of the hypocotyl did not appear to induce any significant changes in the chemical composition of xylem root pressure sap (Fig. 6). The concentrations of potassium (5.6 ± 0.4 mM), sodium (24 ± 5 μM), calcium (2.8 ± 0.3 mM), magnesium (1.2 ± 0.1 mM), phosphorus (908.2 ± 65.7 μM), sulphur (0.8 ± 0.1 mM) and amino acids (22.4 ± 1.9 mM) in the xylem exudates did show some variation, but the differences were not significant (Fig. 6).

In order to study the effect of cold girdling on the chemical composition of stem tissue at the site of cold girdling (III), and on two sections above (I–II) and one section below (IV) the cold girdle (see Fig. 1a), corresponding stem pieces were harvested after 3 d of cold treatment and subsequently analysed. The concentrations of total carbon (35.3 ± 0.2 mmol g^{-1} DW), total nitrogen (0.50 ± 0.02 mmol g^{-1} DW), total sulphur (42.2 ± 2.8 μmol g^{-1} DW), total phosphorus (50.8 ± 2.0 μmol g^{-1} DW), calcium (396 ± 24 μmol g^{-1} DW), and potassium (357 ± 37 μmol g^{-1} DW) were not significantly affected by the 3 d of cold treatment (Fig. 7).

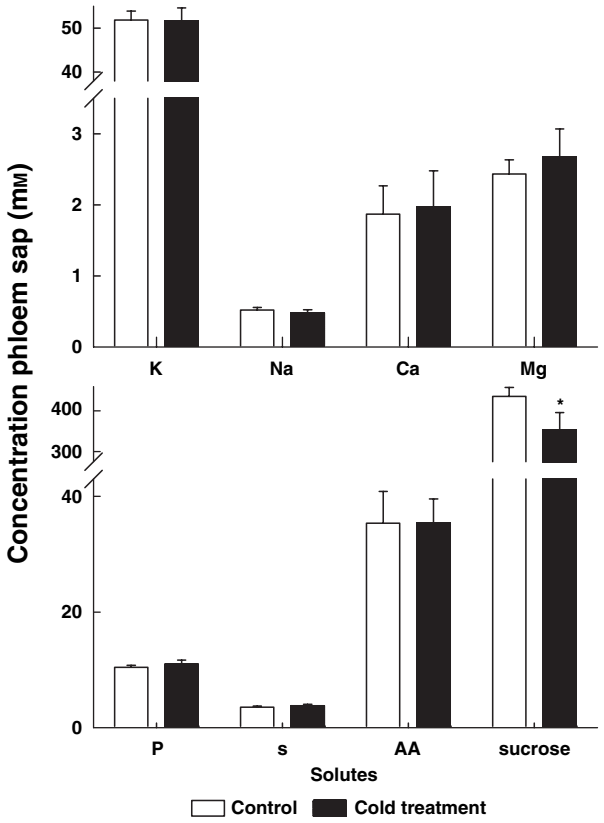

Figure 5. The chemical composition of phloem saps in 45–55-day-old *Ricinus communis*, collected at the hypocotyl after 3 d cold-girdling (□) and in the controls (■). Shown are the mean values of 13–15 replicates, standard errors are indicated by bars.

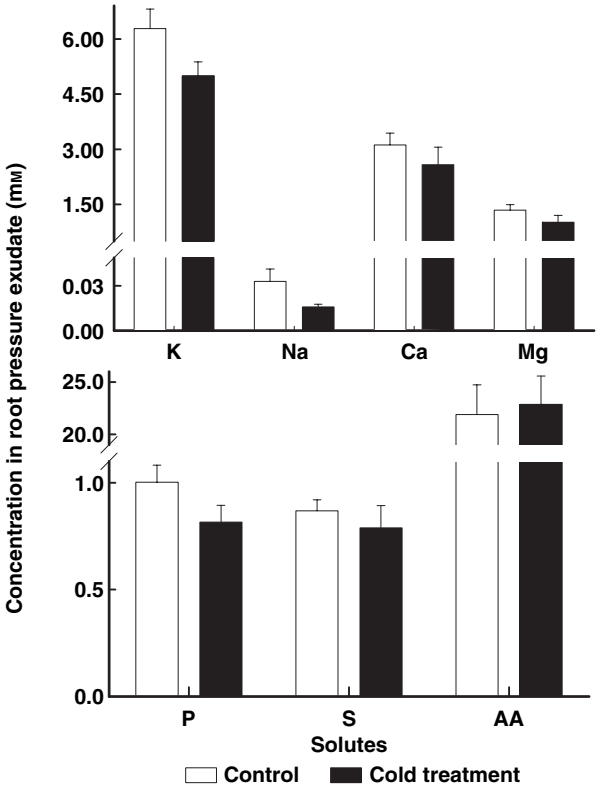

Figure 6. The chemical composition of xylem root pressure saps in 45–55-day-old *Ricinus communis*, collected at the hypocotyl after 3 d cold-girdling (□) and in the controls (■). Shown are the mean values of 13–15 replicates, standard errors are indicated by bars.

The concentrations of soluble carbohydrates and starch on the other hand showed a significant increase in some of the stem segments. An increase became visible, but was not yet significant, in the segment that was subjected to the cold-girdle (III), and increased acropetally which may be due to 'transport' cold treatment *via* the transpiration stream. In the first segment above the cold girdle (II), sugars increased markedly from 765 ± 124 to 1182 ± 310 μmol g^{-1} DW and starch from 100 ± 30 to 210 ± 179 μequival. g^{-1} DW. In the second segment above the cold girdle (I) only the increase in starch concentration (from 73 ± 39 to 179 ± 189 μmol g^{-1} DW) was significant, even though a small increase in sugar concentration was still visible. In the segment below the cold girdle (IV) no changes in chemical composition were detected.

NMR-measurements of water flow in the phloem

In order to measure the effect of cold-girdling on the transport of water in the phloem, quantitative NMR flow imaging was performed on the stem of the plant, below the site of cold girdling (see Fig. 1). Every flow measurement took 30 min, effectively giving an average value for the average flow velocity and flowing volume during the whole measurement period. During every phase of the experiment (before, during and after cold treatment) four measurements were taken, with two waiting periods of 15 min in between to allow the cold girdle to reach the desired temperature.

During the first 30 min of cold girdling, the flow of phloem water was almost completely inhibited. Before the cold treatment was started, an average volume of 4.05 ± 0.29 μL phloem water was moving at an average linear velocity of 0.30 ± 0.02 mm s^{-1} (average of first four measurements; see Fig. 8a & b). During the first 30 min of cold girdling, the amount of flowing water as well as the average linear velocity was reduced sharply. The volume of flowing water was close to zero (0.31 ± 0.35 μL), with a corresponding slight increase in the volume of stationary water. The average linear flow velocity dropped sharply as well (0.10 ± 0.11 mm s^{-1}). The reduction in phloem flow was only temporary, after the first 30 min the movement of phloem water appeared to be restored completely. During the remainder of the cold girdling period, the average flowing volume (3.82 ± 0.14 mm^3) and average linear velocity 0.32 ± 0.02 mm s^{-1}) returned to values comparable with the values measured before cold treatment. Rewarming the cold girdle did not cause any changes in the phloem flow characteristics. The average flowing volume as well as the average linear flow velocity remained at approximately the same level as before cold girdling, even though towards the end of the experiment a slight (but insignificant) reduction of flow velocity and flow volume was observed.

DISCUSSION

Effects of cold girdling on phloem solute concentrations

Remarkably, it was possible to harvest phloem sap by shallow incision into the bark before, during and after cold girdling, even though our NMR flow measurements showed an almost complete stoppage of phloem flow during the first 30 min of cold treatment. Apparently, although during this period phloem flow was severely inhibited, phloem pressure was still high enough for phloem sap to exude from the hypocotyls upon cutting. The evaluated ion and element concentrations in phloem saps were typical and comparable with earlier observations in legumes and *Ricinus* (Pate *et al*. 1974; Jeschke *et al*. 1996; Peuke *et al*. 2001). The most striking observation with regard to changes in the chemical composition in the phloem sap in the present study was the fast and reversible decrease of sucrose concentration during cold girdling. This effect occurred directly after starting the cold treatment, was reversible after finishing the treatment (with a time resolution of about 15–20 min for collecting saps samples in short-term experiments, Fig. 4) and was still measured after 3 d, which was the longest observation time (long-term experiments, Fig. 5). The concentrations of the other phloem sap solutes measured were not affected by the cold treatment. After stopping the cold treatment in the short term experiments, phloem sap sucrose concentration immediately recovered to the same concentration as before cold treatment. The

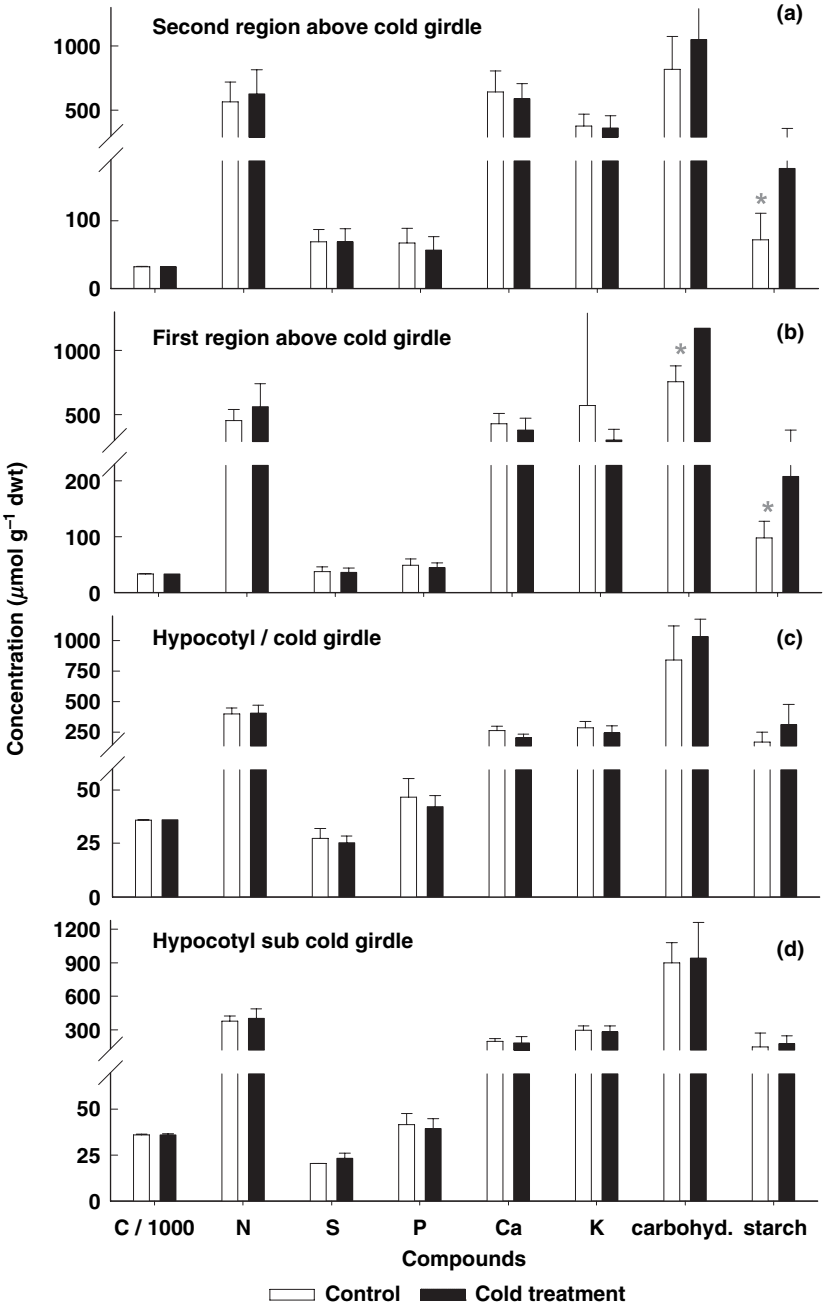

Figure 7. The chemical composition of the axis tissue of 45–55-day-old *Ricinus communis*, harvested after 3 d of cold treatment. Shown are the concentrations in the region of the cold-girdle at the hypocotyl (c) and in the section of the hypocotyl below (d) as well as in the next acropetal sections one (b) and two (a). An average value of nine replicates ±SE.

present observed decrease in sucrose in phloem sap due to cold treatment was similar to that due to the offset of light (Peuke *et al*. 2001). In both cases the solute changes produced no effect on transport velocity in the phloem.

We propose that due the lower temperature, the retrieval of sugar into the phloem after leaching (van Bel 1993, 2003a, b; Lalonde *et al*. 2003) is inhibited. The missing effect of cold treatment on minerals in phloem sap can be correlated to a missing downhill concentration gradient comparing solutes in the phloem with the surrounding tissue (compare data Fig. 5 with Fig. 7). The leached sugars accumulated partly in stem tissue or were converted to starch in the stem parts above the cold girdle (see Fig. 7). Conversion of sugars to starch for regulation of cytosolic sugar levels in source tissues of the phloem is a known response to maintain equilibrium and constant phloem transport (Komor 2000). Additionally, the observed accumulation of released sugars in the form of starch may maintain the downhill sugar concentration gradient from phloem vessels to surrounding tissue, thus providing the basis for continued passive leaching. Minchin & Thorpe (1984) detected an apoplastic sucrose concentration gradient in the stem of bean along the transport pathway. They observed that washout from the apoplast increased when transport was inhibited by manipulating the temperature. Lang & Minchin (1986) assumed that sucrose transporters were

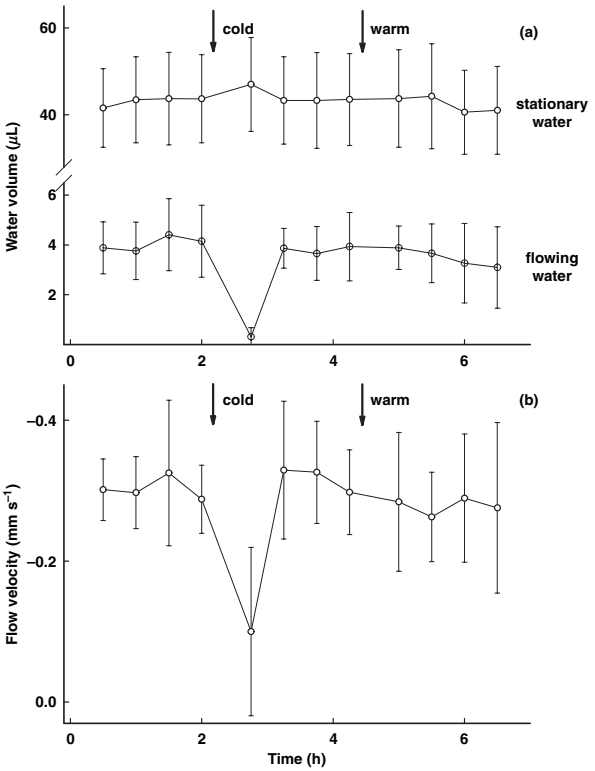

Figure 8. Volume of stationary and flowing water (a) and average linear flow velocity (b) in the transport phloem of 45–55-day-old *Ricinus communis*, measured at the hypocotyl by means of NMR flow imaging. Measurements were taken before and after starting the cold treatment (↓ cold), and during the recovery period after rewarming (↓ warm/RT).

inhibited by cold. One possible candidate for such a transporter would be the sucrose carrier RcSCR1, which was recently found to be involved in sucrose retrieval, but not loading, in hypocotyls of *Ricinus* (Eisenbarth & Weig 2005). Starch accumulation above the cold girdle has been observed before in bean (Hannah *et al.* 2001). Cold-inhibited phloem translocation affected the partitioning of photo-assimilates (Grusak & Minchin 1989; Candolfi-Vasconcelos *et al.* 1994). The composition of the xylem was not affected by the cold treatment in the present study.

Effects of cold girdling on mass flow in the phloem

The presently observed inhibition of sucrose transport in the phloem by cold treatment in *Ricinus* is an effect often found in other plants (see Introduction). But inhibition of translocation in the phloem by chilling was not found in all species. For example bean is sensitive whereas sugar beet is tolerant against chilling (Giaquinta & Geiger 1973). Lang & Minchin (1986) correlated the sensitivity of plant species to the P-protein and explained the chilling effect more as a perturbation of membrane function than as a blocking of sieve plates. In contrast, Giaquinta & Geiger (1973) concluded that blockage of sieve plates rather than inhibited metabolic processes are responsible.

The average phloem flow velocities that were observed in the current study before cold girdling (0.29 ± 0.07 mm s^{-1}) were very similar to those observed before in *Ricinus* by means of NMR flow imaging (0.250 ± 0.004 mm s^{-1}, Peuke *et al.* 2001; 0.25 ± 0.03 mm s^{-1}, C.W. Windt *et al.* unpublished data) and ^{14}C labelling (0.233 mm s^{-1}; Hall, Baker & Milburn 1971). The higher flow rates and average velocities in 3-day-old *Ricinus* seedlings that were observed previously (2.1 ± 0.1 m h^{-1} Köckenberger *et al.* 1997) may have been caused by the special conditions in these very young plants (low transpiration, supply by endosperm), but may also originate from an overestimation of the average flow velocity, caused by the particular flow quantification method that was used (for details contact C.W. Windt *et al.* unpublished data).

In the present study, phloem sap flow was only impeded during the first 30 min of cold treatment. After the first 30 min of cold girdling the flow of phloem water recovered completely, and during the remainder of the cold treatment, as well as after rewarming, the average phloem flow velocities were comparable with those before cold girdling. Similarly, Gould *et al.* (2004) observed a very fast (90 s) and reversible hydrostatic pressure increase in phloem vessels upstream following cold treatment in *Sonchus* which pointed to a reversible inhibition of mass flow at the site of sieve elements.

Good candidates for a fast, short but reversible inhibition of phloem transport by cold treatment may be parietal crystalline P proteins. Displacements of crystalline proteins in the sieve tubes were observed to be involved in effects of phloem chilling before (Giaquinta & Geiger 1973) and the sensitivity of some plant species to chilling was correlated with the occurrence of P proteins (Lang & Minchin 1986). Knoblauch & van Bel (1998) described two major occlusion mechanisms of sieve plates: as a result of laser light (low damage) parietal proteins form a network and secondly, mechanical damage caused P plastids to explode and form a massive plug. The reversible, rapid (< 1 s) closure of the phloem pathway by dispersal of crystalline P proteins in *Fabaceae* was signalled by divalent cations, particularly free calcium (Knoblauch *et al.* 2001). Pickard & Minchin (1990) found some support for the role of free calcium in signalling cold inhibition in phloem transport. In general, cold shock can be signalled by free calcium in plants (as well as heat, osmotic or touch, see review Sanders, Brownlee & Harper 1999) and this would effect sealing by dispersal of P proteins or callose as well. In the present study, total calcium tends to increase in phloem sap and tends to decrease in xylem sap and stem tissue (Figs 5–7), but total concentration of Ca is not relevant for signalling processes. P proteins play a prominent role in sealing sieve tubes and may be a 'temporary line of defence' compared to the 'definitive nature' of callose (van Bel 2003a). However, Knoblauch *et al.* (2001) suggest that phloem pathway control regulated by the effect of calcium on phloem-specific proteins can not be generalized. They, and others,

have also shown that these structures and effects do not occur in other species and families (Eckardt 2001). Although, such an explanation appears adequate in this or similar cases (Gould *et al*. 2004), the question remains as to whether this effect really explains the present observations. Theoretical considerations and mathematical models show that physical factors like temperature, viscosity and elasticity of cell walls have a remarkable effect on phloem flow (Henton *et al*. 2002; Thompson & Holbrook 2003). From Poiseulle's law it follows that the resistance to flow in a phloem vessel will change in accordance with temperature as well as viscosity. Bancal & Soltani (2002) employed an empirically determined formula to estimate the variation of relative resistance ${}^{T}R$ of pure water due to temperature with an error of less than 1%, within a temperature range of 0–42 °C:

$$ {}^{T}R = {}^{20}R\ (-1.208 \times 10^{-5}T3 + 1.432 \times 10^{-3}T^{2} - 0.07318T + 2.07) \quad (1) $$

During our cold girdling experiments the stem temperature was locally lowered from 25 to 0 °C over a length of approximately 4 cm. Using Eqn 1, we can speculate that the relative resistance in the cold girdling region must have increased dramatically, from 95.0% at 25 °C to 193% at 0 °C. Unfortunately, data on real changes in phloem sap viscosity are not available.

Again using an equation by Bancal & Soltani (2002), the following polynomial relationship fits the effect of sucrose concentration on the relative resistance ${}^{S}R$ with an error of less that 1% and for a sucrose concentration (S) range of 0–1.5 M:

$$ {}^{S}R = {}^{3}R\ (0.685S^{4} - 1.0411S^{3} + 0.9513S^{2} + 0.1364S + 0.3396) \quad (2) $$

During our short-term cold girdling experiments, we found a phloem sucrose concentration of approximately 0.42 M both before and after cold treatments, and a phloem sucrose content of approximately 0.37 M during cold treatments. Substituting these values into Eqn 2, this translates to a relative resistance of 45.2% before and after cold treatment, and 43.0% during cold treatment. Speculating once again, this would cause a decrease in relative resistance of 4.8%. During the long-term cooling treatment the sucrose concentration difference was even greater.

In comparison with the doubling of local flow resistance caused by cooling, the reduction of flow resistance by lowering sucrose concentration is small. But if we assume that (1) the whole phloem pathway downstream of the cold girdle is experiencing a lower sucrose concentration than before cold girdling, and that (2) the flow resistance in the last stretch of phloem in the roots is approximately equal to the value of the resistance in the transport phloem in the 30 cm long stem, then the modest reduction in relative flow resistance caused by the reduction in sucrose concentration would be sufficient to counteract the increase in flow resistance caused by cooling. The lowered phloem sap sucrose concentrations that are commonly observed during cold girdling experiments may thus have an adaptive value.

Concluding remarks: how can the effects of cold girdling be explained?

For the observations that have been made during the cold girdling experiments on the transport phloem several explanations may be available: (a) total stoppage of mass/bulk flow and/or (b) leaching of sugars from phloem vessels ('leaky tube' or 'retrieval inhibition' hypothesis). Additionally, it can be speculated about multiple effects of cold on flow resistance by viscosity and sugar concentrations and disturbance of hydrostatic pressure relations.

Ad (a): in the present study it was shown for the first time that water flow was totally stopped in the phloem by cold treatment, but this effect lasted only a few minutes and recovered afterwards while the cold treatment persisted. The blockage of sieve plates by dispersal of parietal crystalline P proteins may provide an explanation.

Ad (b): release of sugars along the phloem pathway and inhibition of sugar retrieval by low temperatures may occur. Accordingly, it was observed that sugar concentrations in phloem sap decreased downstream during girdling (short-term as well as long-term experiments) and that starch accumulated above the cold girdle, obviously after conversion from sucrose. Upon re-warming sugar concentration in phloem sap recovered immediately.

Additionally, cold girdling may locally change flow resistances in the phloem. On the one hand, it could be that flow resistance increased at the site of cold treatment because of the effects of temperature on phloem sap viscosity. On the other hand, flow resistance may be decreased downstream of the cold girdle as a result of a decrease in sucrose concentration and the associated decrease in phloem sap viscosity. It is therefore possible to speculate that the decrease in phloem flow resistance downstream of the cold girdle may have been sufficient to wholly or partially cancel out the increase in flow resistance that was caused by the effect of temperature on viscosity.

ACKNOWLEDGMENT

This paper was financially supported by European Commission Large-Scale Facility Project HPRI-CT-1999–00085 1-3- 2000-1-3- 2003.

REFERENCES

Bancal P. & Soltani F. (2002) Source-sink partitioning. Do we need Münch? *Journal of Experimental Botany* **53**, 1919–1928.

van Bel A.J.E. (1993) The transport phloem. Specifics of its functioning. *Progress in Botany* **54**, 134–150.

van Bel A.J.E. (2003a) The phloem, a miracle of ingenuity. *Plant, Cell and Environment* **26**, 125–149.

van Bel A.J.E. (2003b) Transport phloem: low profile, high impact. *Plant Physiology* **131**, 1509–1510.

Candolfi-Vasconcelos M.C., Candolfi M.P. & Koblet W. (1994) Retranslocation of carbon reserves from the woody storage tissues into the fruit as a response to defoliation stress during the ripening period in *Vitis vinifera* L. *Planta* **192**, 567–573.

Eckardt N.A. (2001) A calcium-regulated gatekeeper in phloem sieve tubes. *Plant Cell* **13**, 989–992.

Eisenbarth D.A. & Weig A.R. (2005) Sucrose carrier RcSCR1 is involved in sucrose retrieval, but not in sucrose unloading in growing hypocotyls of *Ricinus communis* L. *Plant Biology* **7**, 98–103.

Giaquinta R.T. & Geiger D.R. (1973) Mechanism of inhibition of translocation by localized chilling. *Plant Physiology* **51**, 372–377.

Gould N., Minchin P.E.H. & Thorpe M.R. (2004) Direct measurements of sieve element hydrostatic pressure reveal strong regulation after pathway blockage. *Functional Plant Biology* **31**, 987–993.

Grusak M.A. & Minchin P.E.H. (1989) Cold-inhibited phloem translocation in sugar beet. IV. Analysis of the cooling-induced repartitioning hypothesis. *Journal of Experimental Botany* **40**, 215–223.

Hall S.M., Baker D.A. & Milburn J.A. (1971) Phloem transport of ^{14}C labelled assimilates in *Ricinus*. *Planta* **100**, 200–207.

Hannah M.A., Iqbal M.J. & Sanders F.E. (2001) Adaptation to long-term cold-girdling in genotypes of common bean (*Phaseolus vulgaris* L.). *Journal of Experimental Botany* **52**, 1123–1127.

Henton S.M., Greaves A.J., Piller G.J. & Minchin P.E.H. (2002) Revisiting the Münch pressure–flow hypothesis for long-distance transport of carbohydrates: modelling the dynamics of solute transport inside a semipermeable tube. *Journal of Experimental Botany* **53**, 1411–1419.

Jenner C.F., Xia Y., Eccles C.D. & Callaghan P.T. (1988) Circulation of water within wheat grain revealed by nuclear magnetic resonance micro-imaging. *Nature* **336**, 399–402.

Jeschke W.D., Peuke A.D., Kirkby E.A., Pate J.S. & Hartung W. (1996) Effects of P deficiency on the uptake, flows and utilization of C, N and H_2O within intact plants of *Ricinus communis* L. *Journal of Experimental Botany* **47**, 1737–1754.

Knoblauch M. & van Bel A.J.E. (1998) Sieve tubes in action. *The Plant Cell* **10**, 35–50.

Knoblauch M., Peters W.S., Ehlers K. & van Bel A.J.E. (2001) Reversible calcium-regulated stopcocks in legume sieve tubes. *The Plant Cell* **13**, 1221–1230.

Köckenberger W., Pope J.M., Xia Y., Jeffrey K.R., Komor E. & Callaghan P.T. (1997) A non-invasive measurement of phloem and xylem water flow in castor bean seedlings by nuclear magnetic resonance microimaging. *Planta* **201**, 53–63.

Komor E. (2000) Source physiology and assimilate transport: the interaction of sucrose metabolism, starch storage and phloem export in source leaves and the effects on sugar status in phloem. *Australian Journal of Plant Physiology* **27**, 497–505.

Kuchenbrod E., Landeck M., Thuermer F., Haase A. & Zimmermann U. (1996) Measurement of water flow in the xylem vessels of intact maize plants using flow-sensitive NMR imaging. *Botanica Acta* **109**, 184–186.

Lalonde S., Tegeder M., Throne-Holst M., Frommer W.B. & Patrick J.W. (2003) Phloem loading and unloading of sugars and amino acids. *Plant, Cell and Environment* **26**, 37–56.

Lang A. & Minchin P.E.H. (1986) Phylogenetic distribution and mechanism of translocation inhibition by chilling. *Journal of Experimental Botany* **37**, 389–398.

Minchin P.E.H. & Thorpe M.R. (1984) Apoplastic phloem unloading in the stem of bean. *Journal of Experimental Botany* **35**, 538–550.

Oparka K.J. & Turgeon R. (1999) Sieve elements and companion cells – traffic control centers of the phloem. *The Plant Cell* **11**, 739–750.

Pate J.S., Sharkey P.J. & Lewis O.A.M. (1974) Phloem bleeding from legume fruits – A technique for study of fruit nutrition. *Planta* **120**, 229–243.

Patrick J.W. (1997) Phloem unloading: sieve element unloading and post-sieve element transport. *Annual Review of Plant Physiology and Plant Molecular Biology* **48**, 191–222.

Peuke A.D., Rokitta M., Zimmermann U., Schreiber L. & Haase A. (2001) Simultaneous measurement of water flow velocity and solute transport in xylem and phloem of adult plants of *Ricinus communis* during day time course by nuclear magnetic resonance (NMR) spectrometry. *Plant, Cell and Environment* **24**, 491–503.

Pickard W.P. & Minchin P.E.H. (1990) The transient inhibition of phloem translocation in *Phaseolus vulgaris* by abrupt temperature drops, vibration, and electric shock. *Journal of Experimental Botany* **41**, 1361–1369.

Reinders J.E.A., Van As H. & Schaafsma T.J. (1988) water-balance in cucumis plants, measured by nuclear magnetic-resonance 1. *Journal of Experimental Botany* **39**, 1199–1210.

Roe J.H. (1955) The determination of sugar in blood and spinal fluid with anthron reagent. *Journal of Biological Chemistry* **212**, 335–342.

Rokitta M., Zimmermann U. & Haase A. (1999) Fast NMR flow measurements in plants using FLASH imaging. *Journal of Magnetic Resonance* **137**, 29–33.

Sanders D., Brownlee C. & Harper J.F. (1999) Communicating with Calcium. *The Plant Cell* **11**, 691–706.

Schaafsma T.J., Van As H. & Palstra W.D. (1992) Quantitative measurement and imaging of transport processes in plants and porous-media by H-1-NMR. *Magnetic Resonance Imaging* **10**, 827–836.

Scheenen T.W., van Dusschoten D., De Jager P.A. & Van As H. (2000a) Microscopic displacement imaging with pulsed field gradient turbo spin-echo NMR. *Journal of Magnetic Resonance* **142**, 207–215.

Scheenen T.W.J., van Dusschoten D., de Jager P.A. & Van As H. (2000b) Quantification of water transport in plants with NMR imaging. *Journal of Experimental Botany* **51**, 1751–1759.

Scheenen T.W., Vergeldt F.J., Windt C.W., de Jager P.A. & Van As H. (2001) Microscopic imaging of slow flow and diffusion: a pulsed field gradient stimulated echo sequence combined with turbo spin echo imaging. *Journal of Magnetic Resonance* **151**, 94–100.

Thompson M.V. & Holbrook N.M. (2003) Application of a single-solute non-steady-state phloem model to the study of long-distance assimilate transport. *Journal of Theoretical Biology* **220**, 419–455.

Van As H. & Schaafsma T.J. (1984) Noninvasive measurement of plant water flow by nuclear magnetic resonance. *Biophysical Journal* **45**, 469–472.

Zimmermann U., Schneider H., Wegner L.H. & Haase A. (2004) Water ascent in tall trees: does evolution of land plants rely on a highly metastable state? *New Phytologist* **162**, 575–615.

Received 11 February 2005; received in revised form 25 May 2005; accepted for publication 1 June 2005