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Heat girdling does not affect xylem integrity: an *in vivo* magnetic resonance imaging study in the tomato peduncle

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Summary

- Heat girdling is a method to estimate the relative contribution of phloem vs xylem water flow to fruit growth. The heat girdling process is assumed to destroy all living tissues, including the phloem, without affecting xylem conductivity. However, to date, the assumption that xylem is not affected by heat girdling remains unproven.
- In this study, we used *in vivo* magnetic resonance imaging (MRI) velocimetry to test if heat girdling can cause xylem vessels to embolize or affect xylem water flow characteristics in the peduncle of tomato (*Solanum lycopersicum* cv Dirk).
- Anatomical and MRI data indicated that, at the site of girdling, all living tissues were disrupted, but that the functionality of the xylem remained unchanged. MRI velocimetry showed that the volume flow through the secondary xylem was not impeded by heat girdling in either the short or the long term (up to 91 h after girdling).
- This study provides support for the hypothesis that in the tomato peduncle the integrity and functionality of the xylem remain unaffected by heat girdling. It therefore confirms the validity of the heat girdling technique as a means to estimate relative contributions of xylem and phloem water flow to fruit growth.

Introduction

Fleshy fruit growth is the result of the influx of water, minerals and assimilates through xylem and phloem, on the one hand, minus losses through transpiration and respiration on the other (Lang & Thorpe, 1989). While the phloem pathway carries most of the carbon into the fruit, the xylem mainly conducts water with low concentrations of mineral nutrients such as calcium (Sauré, 2005). The relative magnitude of these two inputs determines fruit growth and quality. Quantification of these contributions has been the objective of many studies (e.g. Ho *et al.*, 1987; Lang & Thorpe, 1989; Morandi *et al.*, 2007; Windt *et al.*, 2009; Hanssens *et al.*, 2015), but often produced contrasting results. In tomato, for example, xylem contributions ranging from 10% (Liu *et al.*, 2007) to 75% (Windt *et al.*, 2009) have been reported. These conflicting results might be partially explained by differences in environmental conditions. Hanssens *et al.* (2015) showed that the relative xylem contribution increased significantly when the light intensity was lowered by shading. Unfortunately, many of the techniques that have been used to estimate phloem and xylem influx into fruits have significant drawbacks or are based on questionable assumptions. As a result of this, the debate about the relative contributions of xylem and phloem to fruit growth remains strong (Hubeau & Steppe, 2015).

The dye infusion method is a destructive method to assess xylem connectivity between fruit and plant, whereby an excised peduncle or stem is submerged in xylem-mobile dye while fruit transpiration is stimulated. After a certain time, the fruit is sectioned to assess dye infiltration as an indicator of xylem functionality. Although this technique has been frequently used, especially in grapes (Düring *et al.*, 1987; Keller *et al.*, 2006), it only provides qualitative data and its validity has been questioned owing to inconsistencies when compared with other techniques to estimate xylem functionality (Rogiers *et al.*, 2001).

The mineral accumulation method is used to estimate the relative xylem and phloem contributions to fruit growth. This technique is based on the assumption that all calcium in the fruit is imported through the xylem (Ho *et al.*, 1987). If this assumption holds, the contribution of xylem flow (X) can be estimated from the accumulation of calcium in the fruit (ΔCa_{fr}), provided that the calcium concentration in the xylem ($[Ca]_x$) can be accurately measured:

$$X = \Delta Ca_{fr} / [Ca]_x \quad \text{Eqn 1}$$

A problem with this technique is that the amount of calcium imported through the phloem has to be negligible.

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Unfortunately, multiple studies have shown that phloem tissues can contain a considerable concentration of calcium (Pate *et al.*, 1998; Peuke *et al.*, 2006). Moreover, de Freitas *et al.* (2014) observed that phloem influx might contribute significantly to the calcium content in tomato. These findings suggest that the mineral accumulation technique is not a valid means to estimate the relative contribution of xylem to fruit growth.

The subtractive method (Lang & Thorpe, 1989) is another method for estimating the relative xylem and phloem water flow contributions to growing fruit. In this method, a fruit-bearing peduncle or branch is girdled in order to destroy the living phloem, while leaving the xylem intact. The contribution of xylem flow (X), phloem flow (P) and transpiration (T) can be estimated by comparing the volume change of intact (ΔV_{intact}), girdled ($\Delta V_{girdled}$) and detached ($\Delta V_{detached}$) fruits of proportional size. This volume change can be measured directly using the Archimedes principle (Lang & Thorpe, 1989) or inferred from diameter measurements (e.g. Nordey *et al.*, 2015) It is furthermore assumed that the influx of dry matter is negligible compared with water influx, and that the cumulative changes in fruit volume are hence an integration of the fruit's water inflows and outflows. This leads to the following set of equations on the basis of which all contributions can be estimated:

$$\Delta V_{intact} = X - T + \mathrm{P} \qquad \text{Eqn 2}$$

$$\Delta V_{girdled} = X - T \qquad \text{Eqn 3}$$

$$\Delta V_{detached} = T \qquad \text{Eqn 4}$$

This method has been used on a wide range of fruits and berries, such as apple (Lang, 1990; Lang & Volz, 1998; Morandi *et al.*, 2011), cherry (Athoo *et al.*, 2015; Brüggenwirth *et al.*, 2016), citrus (Garcia-Luis *et al.*, 2002), grape (Lang & Thorpe, 1989; Creasy & Lombard, 1993; Greenspan *et al.*, 1994, 1996; Rogiers *et al.*, 2001), kiwifruit (Clearwater *et al.*, 2009, 2012; Morandi *et al.*, 2012; Torres-Ruiz *et al.*, 2016), mango (Nordey *et al.*, 2015), peach (Morandi *et al.*, 2007, 2010), pear (Morandi *et al.*, 2014a,b) and tomato (Else *et al.*, 1996; Plaut *et al.*, 2004; Guichard *et al.*, 2005; Jan & Kawabata, 2011; de Freitas *et al.*, 2014; Hanssens *et al.*, 2015). While in woody tissue, girdling can be achieved by mechanical removal of the bark, the same is not always feasible for herbaceous pedicels and peduncles. The girdling of the pedicel or peduncle can then be achieved by the application of heat, through steam (Lang & Thorpe, 1989), hot water poured over the tissue (Jan & Kawabata, 2011), or an electrical current applied to a resistance attached to the tissue (Creasy & Lombard, 1993; Greenspan *et al.*, 1994; Else *et al.*, 1996; Guichard *et al.*, 2005; Clearwater *et al.*, 2012; de Freitas *et al.*, 2014; Hanssens *et al.*, 2015). As the latter method is easily reproducible and controllable, it has most often been used when mechanical girdling is impossible or impractical.

Despite its widespread use, the validity of the heat girdling method has been questioned (e.g. Fishman *et al.*, 2001; Windt *et al.*, 2009). The heat girdling method is based on the assumption that when the phloem is destroyed, the xylem remains intact and its functionality and resistance will remain unchanged. It has been argued, however, that destruction of the living tissues in the stem might expose the xylem to the air and cause xylem embolism formation, or change the flow resistance in the xylem indirectly as a result of the release of cell contents into the xylem (Windt *et al.*, 2009) or the cessation of ionic exchange between xylem and phloem (Zwieniecki *et al.*, 2004). While the release of cell contents into the xylem might decrease xylem resistance as a result of ionic effects, the cessation in ionic exchange might increase it (Zwieniecki *et al.*, 2001, 2004).

So far, there is no conclusive proof for the validity and accuracy of the girdling technique, and many of the authors who apply the technique acknowledge that it may induce errors (Hanssens *et al.*, 2015; Nordey *et al.*, 2015; Torres-Ruiz *et al.*, 2016). The extent of these possible errors has been assessed by a theoretical fruit growth model for peach (Fishman *et al.*, 2001) and others have used a microscopic study on tomato peduncles to ensure that the xylem remains intact (Guichard *et al.*, 2005). More recently, xylem function after girdling was assessed in grape peduncles with a dye-uptake experiment (Keller *et al.*, 2015), yet this only provided a qualitative indication of xylem functioning without being able to ensure that the total amount of xylem flow was unaltered. Hence, no conclusive experimental data showing the quantitative effects on xylem functionality have been presented so far.

The only way to measure xylem and phloem flow *in vivo* and nondestructively is by means of medical imaging techniques such as positron emission tomography (PET), which is able to measure carbon fluxes (Hubeau & Steppe, 2015), or magnetic resonance imaging (MRI), which measures water (Borisjuk *et al.*, 2012). The latter has been shown to be a useful tool to simultaneously measure xylem and phloem water transport (Windt *et al.*, 2006), and can provide data on both volume flow and flow-conducting area on a per-pixel basis (Scheenen *et al.*, 2000; Van As, 2007). Its applicability has also been shown in tomato peduncles (Windt *et al.*, 2009). While this technique is capable of nondestructively measuring anatomy as well as water transport, its widespread application has so far been limited by the complexity, cost and size of the equipment. Smaller and portable MRI devices have recently been developed that promise to resolve some of these methodological constraints (Windt & Blümler, 2015), and other groups have shown proof of principle that even large, custom-made, stationary MRI devices can be placed outside to measure flow in trees (Nagata *et al.*, 2016). However, in both cases the devices should still be regarded as prototypes, and field applications therefore remain limited.

Because MRI provides unique opportunities for nondestructive and highly spatially resolved measurements, it is particularly suited for validation of other techniques. In this study, we used MRI, combined with histology, to test whether the xylem suffers from direct or indirect functional damage as a result of heat girdling. In this way, we aim to validate the subtractive method for estimating xylem and phloem water flow contributions to fruit growth. To our knowledge, this is the first time that this widely used method has been qualitatively and quantitatively tested.

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Materials and Methods

Plant material and experimental setup

Measurements were conducted on the peduncle of the first truss of four tomato plants (*Solanum lycopersicum* L. cv Dirk). Plants were grown in a small glasshouse compartment at the faculty of Bioscience Engineering, Ghent University, Belgium. All plants were sown in rockwool blocks (Grodan Delta; Grodan, Roermond, the Netherlands) on 9 May 2014. These blocks were transplanted onto rockwool slabs (Grodan Vital; Grodan) around the time the first trusses started to develop (30 June). The rockwool slabs with the plants were transported to the MRI facilities at Forschungszentrum Jülich, Germany, on 25 July, where measurements started on 30 July allowing the plants 5 d to recover from the transport. Characteristics of the measured trusses are summarized in Table 1. A trickle irrigation system provided the plants with nutrient solution (electric conductivity *c.* 2.7 mS cm^{-1}) up until the measurements. Owing to practical constraints, plants were manually watered with the same nutrient solution when they were placed in the imager. As a result of this practical constraint, the plant bearing truss 1 was involuntarily subjected to slight drought stress before the girdling treatment. The possible consequences of this drought are discussed later. In the imager, a continuous light intensity of 300 μmol m^{-2} s^{-1} was provided by a 300 W metal-halide lamp. The temperature was kept at 20°C at all times.

Anatomical reference MRI images and flow maps were constructed for the study truss of each plant before girdling. After girdling, a second set of anatomical reference MRI images and flow maps were constructed. Flow measurements after girdling were done twice for trusses 1 and 2. Trusses 3 and 4 were kept in the imager for 4 and 2 d, respectively, while repeating flow measurements every 170 min. During this prolonged period, lights were continuously kept on to ensure constant environmental conditions.

Girdling technique

Heat girdling of the peduncle was performed according to the technique of Guichard *et al.* (2005). An insulated constantan wire (length, 0.5 m; diameter, 0.25 mm; resistance, 4.8 Ω) was tightly coiled around the peduncle over a length of at least 1 cm and heated by means of an electrical current (3.5 V) for 3 min (Fig. 1). This way, the temperature of the peduncle was raised to *c.* 75°C (monitored beneath the constantan wire coil with a thermocouple (Type K; Keysight Technologies, Santa Rosa, CA, USA)). During the measurements, the trusses were supported by loosely connecting them to the main stem with a piece of cord to avoid mechanical damage to the xylem by buckling of the peduncle.

Table 1 Characteristics of each tomato truss at the respective time of flow measurement (DAA, d after anthesis of the first fruit on the truss)

	Truss 1	Truss 2	Truss 3	Truss 4
Plant age (d)	82	83	84	89
DAA	24	28	24	28
No. of fruits	5	5	4	4

Fig. 1 (a, b) Photographs showing the insulated constantan wire coiled around the tomato peduncle to perform the heat girdling (a) and the result of heat girdling on the tomato peduncle (b).

MRI velocimetry

Hardware Magnetic resonance imaging velocimetry was done at the IBG-2 plant sciences institute at the Forschungszentrum Jülich. A Varian spectrometer was employed, comprising a 4 kW RF amplifier, a set of Copley 282 400A gradient amplifiers (Copley Controls, Canton, MA, USA) and a custom-built 1.5 T split-bore superconducting magnet (Magnex Scientific, Oxford, UK). The magnet was based on a standard, 94-cm-bore tubular medical MRI magnet, in which the middle of the tubular structure was removed to create a 50 cm vertical gap, allowing objects of up to 4.5 m tall to be placed vertically inside the magnet. The centre of the magnet was fitted with a custom built biplanar gradient set, consisting of two 49-cm-gradient disks with a 12 cm gap and a maximum gradient strength of 800 mT m^{-1} (Tesla Engineering, Storrington, UK). To excite the sample and to receive the nuclear magnetic resonance (NMR) signal, a custom-built 10-turn solenoidal radiofrequency (RF) coil was used. The coil was handmade by winding silver-plated 0.5 mm copper wire

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into the grooves of a Teflon spindle. The hollow spindle consisted of two halves, which were mounted onto the truss stalk before winding the coil. After completion, the RF coil was connected to a detachable tuning module.

MRI: velocimetry and data processing Flow imaging measurement was done using a pulsed field gradient–stimulated echo–multi spin echo sequence (PFG-STE-MSE), based on the sequence described by Windt *et al.* (2007). The following experimental parameters were employed: field of view (FOV), 11 × 11 mm (trusses 1–3) or 10 × 10 mm (truss 4); slice thickness, 3 mm; matrix size, 128 × 128 pixels; repetition time, 2.5 s; no averaging; spectral width, 50 kHz; echo time, 4.69 ms; flow encoding, 32 Q steps; flow labelling time (Δ), 40 ms; PFG duration (δ), 3 ms; PFG_{max}, 400 mT m^{-1}.

By analysing the PFG-STE-MSE measurements as described by Scheenen *et al.* (2000), we were able to construct flow maps of phloem and xylem water flow into the truss. Using the reference tubes, which represent stationary water, for calibration, the flow measurements were processed to yield quantitative flow maps representing the amount of stationary water per pixel, the amount of flowing water or flow-conducting area per pixel, the average linear velocity of the flowing water and the average volume flow per pixel. The position and shape of the phloem and xylem flow maps correspond closely with the position of the xylem, external phloem and perimedullary regions that were visible in the anatomical reference provided by the amplitude and T_2 maps (see later Fig. 5a, before girdling, and 5c, after girdling).

MRI: amplitude–T_2 mapping Amplitude–T_2 maps were acquired by means of a Carr Purcell Meiboom Gill (CPMG) sequence. The measurements were done using the following settings: FOV, 11 × 11 mm (trusses 1–3) or 10 × 10 mm (truss 4); slice thickness, 3 mm; matrix size, 256 × 256; number of averages, 2; echo time, 4 ms; number of echoes, 32; repetition time, 5 s; spectral width, 50 kHz. The acquired datasets were processed using fitting routines written in IDL (Research Systems Inc., Boulder, CO, USA). The datasets were fitted on a per-pixel basis, using a monoexponential decay function (van der Weerd *et al.*, 2000), which yields quantitative maps of amplitude and T_2 (Edzes *et al.*, 2000).

Anatomical analysis

After the MRI measurements, all peduncles were cut and preserved in 70% v/v ethanol. Peduncle segments measuring 0.5 cm in height were thoroughly rinsed in demineralized water and glued onto the vibratome stage using superglue (Loctite 406, Henkel, Belgium). Sections 40–50 μm thick were prepared with a vibrating microtome (HM 650V; Thermo Scientific, Dreieich, Germany). Sections were taken at the location of girdling as well as upstream and downstream from the location of girdling, at a distance of between 1 and 1.5 cm of the damaged zone. After a short treatment with commercial bleach (5% v/v sodium hypochlorite), sections were triple-stained in 1% w/v astrablue, 1% w/v chrysoidine and 1% w/v acridine red. After rinsing in 2-propanol, sections were mounted in Euparal (Carl Roth, Karlsruhe, Germany). Slides were observed and photographed with a Nikon E600 microscope equipped with a Nikon DXM1200 camera.

Statistical analysis

Pre- and post-girdling data were compared using a paired *t*-test. Statistical analyses were carried out in SIGMAPLOT 12 (Systat Software Inc., Chicago, IL, USA).

Results

Effects of girdling on peduncle anatomy

The effect of girdling on the living tissues of the peduncle structure is shown in Fig. 2. Above (Fig. 2a,b) and below (Fig. 2e,f) the girdled zone, tissues appear physically intact and easily distinguishable. At the location of girdling, the epidermal and cortical tissues, the latter comprising parenchyma and collenchyma, are disrupted and compressed, as well as the phloem and cambium (Fig. 2c,d). Furthermore, the internal phloem bundles, situated in the perimedullary region, which are often associated with a single, relatively large xylem vessel, as well as pith parenchyma did not escape the detrimental effects of applied heat and are also compressed and disrupted (Fig. 2c). Tissues and cell types with lignified secondary cell walls, which are stained red, remained physically intact (Fig. 2c,d). These include sclerenchyma fibres that are located between compressed phloem and cortical tissues (Fig. 2d), the ring composed of secondary xylem (transport elements and other sclerified cells such as xylem fibres), which contained no parenchyma cells, as well as the single internal xylem vessels located in the phloem bundles of the perimedullary region (Fig. 2c). The effect of girdling was consistent for all treated peduncles (Supporting Information Fig. S1).

The MRI amplitude maps (Fig. 3) confirm that heat girdling destroyed all living tissues in the tomato peduncle, and only the secondary xylem remained hydrated after girdling. The living tissues were not only disrupted, but also quickly lost all water and became invisible on the MRI amplitude maps (Fig. 3, right column). The xylem vessels in the perimedullary region remained intact and visible under light microscopy (Fig. S1), but lost their function and became dehydrated. As the perimedullary region mainly comprises phloem (Fig. 4), and the few xylem vessels that were present became dehydrated, this region became invisible on the MRI amplitude maps after girdling.

Fig. 3 further shows not only that the secondary xylem was physically intact, but also that girdling did not cause cavitation in the xylem vessels. Embolized vessels become visible in the figure as black pixels within the xylem tissue. The black pixels that are visible in the post-girdling images correspond to the black pixels in the pregirdling images, showing that these are native embolisms that were already present before the heat girdling treatment. In truss 3 only, cavitation became slightly more pronounced after girdling than before (36 discernible locations of embolism vs 28 before girdling). An area containing a large

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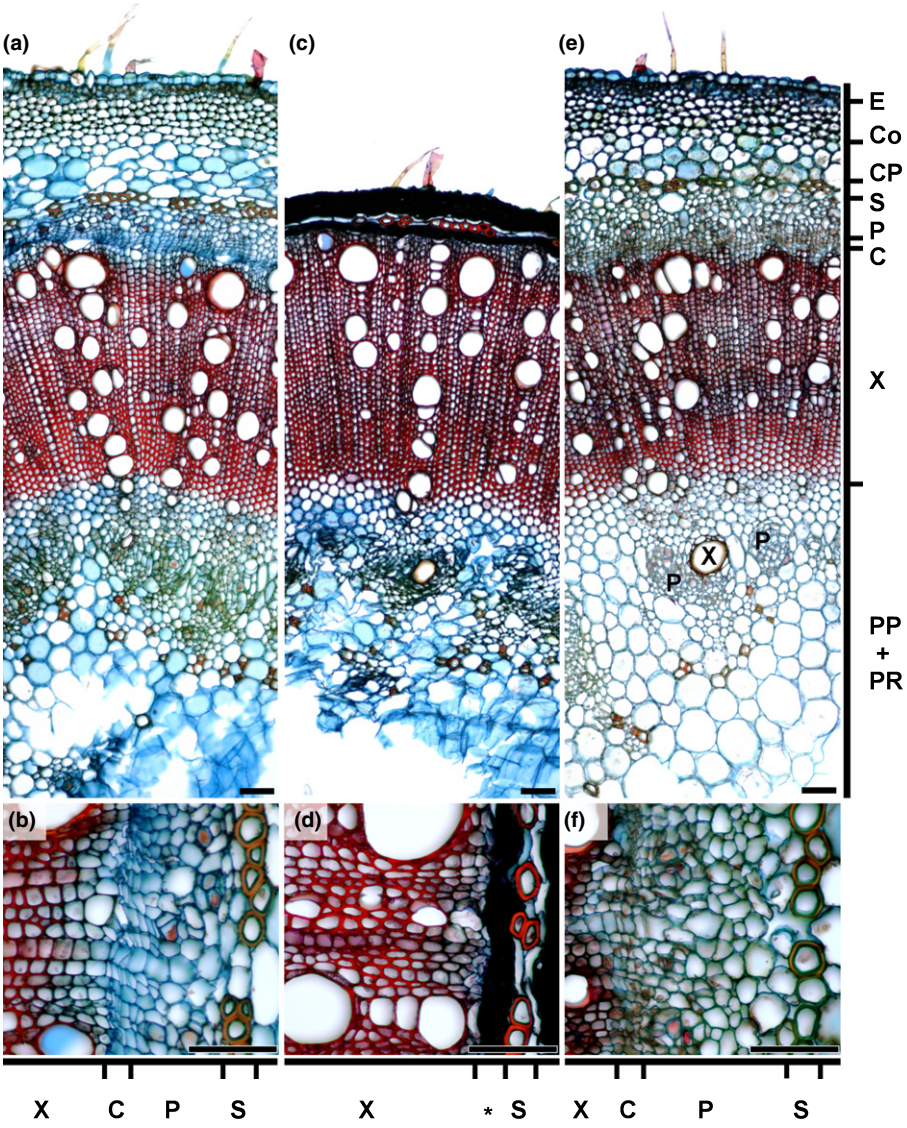

Fig. 2 Anatomy of the tomato peduncle of truss 4, visualized by light microscopy: (a, b) downstream from the location of girdling; (c, d) at the location of girdling; and (e, f) upstream from the location of girdling. Lower panels are detailed micrographs of the peduncle anatomy near the cambium. E, epidermis; Co, collenchyma; CP, cortex parenchyma; S, sclerenchyma fibres; P, phloem; C, cambium; X, xylem; PP, pith parenchyma including the perimedullary region (PR) containing X and P; *, compressed tissues including cambium and phloem. Bars, 100 μm.

number of cavitated vessels before girdling was present in this truss, most likely because the peduncle of this truss had already bent to a steep angle as a result of the weight of the tomatoes before the start of the first MRI measurements.

Does girdling affect xylem conductive functionality?

Fig. 5 shows the flow masks before and after girdling for truss 4. Flow-conducting pixels before girdling could be assigned to xylem, phloem, or tissues located in the perimedullary region using the anatomical MRI reference images and are visible as an outer phloem ring, an inner perimedullary ring and a broad intermediate xylem ring (Fig. 5b). Within the perimedullary region, it was not possible to distinguish between xylem and phloem as a result of the resolution of the flow images: the pixel size of the flow images is 7385 μm², while the size of the xylem vessels ranges between 1870 and 4120 μm². After girdling, only the secondary xylem ring still conducted water. The flow pattern in this xylem ring was slightly different after girdling than before girdling, which is reflected by changes in the distribution of the flow-conducting pixels before and after girdling (Fig. 5b,d). This trend was noticeable in the peduncle of all trusses, as seen in the flow maps before and after girdling (Fig. S2). Despite these slight changes in flow pattern, the total xylem volume flow did not significantly change after girdling in trusses 2, 3 and 4 (−2.9%, −6.3% and +8.2%, respectively, Fig. 6), and overall no significant difference was noticed ($P = 0.868$, $n = 3$). Truss 1, however, showed an increase in xylem volume flow of 121%, resulting in a volume flow after girdling that was higher than the total volume flow in xylem, phloem and the perimedullary region combined in the pregirdling measurement. The data from this truss are to be treated with caution, because much time was spent on fine-tuning the spectrometer settings while the first peduncle was measured. The first successful flow image was obtained during the night (during which lights in the imager were kept switched on), > 12 h after the last manual irrigation. Owing to the low water buffering capacity of the rockwool substrate (De Swaef *et al.*, 2012), this might have led to a limited degree of drought

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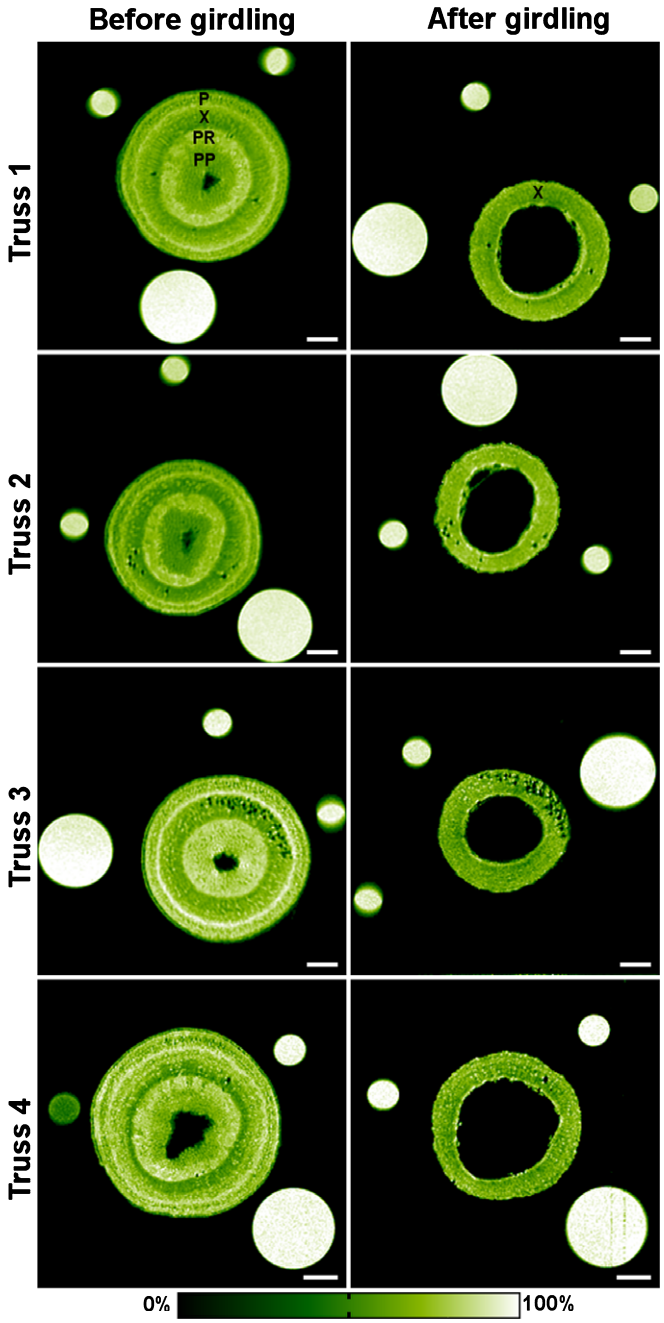

Fig. 3 Water content maps acquired by means of magnetic resonance imaging (MRI) before (left panels) and after (right panels) heat girdling for all tomato trusses. The three reference tubes surrounding each truss are also visible. P, phloem; X, xylem; PR, perimedullary region as a part of the pith parenchyma (PP). Bars, 1 mm.

stress during the measurement before girdling. This is also reflected by the fact that xylem volume flow is markedly smaller in this truss compared with the other trusses. After the heat girdling treatment (and before the first post-girdling measurement) the plant was manually rewatered, which most likely resulted in an increase in xylem flow to the truss.

In trusses 3 and 4, the postgirdling flow measurements were continued for 91 and 32 h, respectively, to investigate the long-term effects of girdling on xylem conductive functionality. During these long-term measurements the rockwool slabs were placed in a dish and allowed access to a few centimetres of nutrient solution to ensure sufficient water supply. While slight fluctuations in xylem flow were present during the day after girdling (truss 4; Fig. 7a), no significant trend was seen in xylem volume flow ($P = 0.98$), even after 3 d (truss 3, $P = 0.74$; Fig. 7b).

Discussion

Secondary xylem functionality

The subtractive method for estimating contributions of xylem and phloem water flow to fruit growth has been widely used, but at the same time, its validity has been questioned (Fishman *et al.*, 2001; Windt *et al.*, 2009). There are a number of reasons why girdling might affect xylem functionality.

First, living tissues are attributed an important role in protecting the xylem against embolism formation by preventing the entry of air (Hacke & Sperry, 2001). While xylem conductance in stems has been shown to be hampered indirectly by feedback inhibition of girdling on photosynthesis and thus stomatal conductance (De Schepper *et al.*, 2010; Bloemen *et al.*, 2013), mechanical girdling of the phloem might also induce a direct wounding effect on the xylem (Zwieniecki *et al.*, 2004). We argue that heat girdling applied in our study is less prone to such xylem wound responses as suggested by Zwieniecki *et al.* (2004), as the phloem is not mechanically removed, but merely disrupted through the application of heat. In this way, there is no danger of cutting into the xylem, and a protective layer of dead tissue is left to shield the xylem from direct exposure to its surroundings. As a result, xylem may be less vulnerable to physical disruption than a mechanically girdled organ, which is suggested by our results indicating unhampered xylem functionality after girdling (Figs 5, 6). Furthermore, heat girdling did not lead to the formation of embolisms in the tomato peduncles (Fig. 3).

A second potential problem with the disruption of living tissue lies in the fact that xylem resistance is known to respond to changes in the ionic content of the xylem sap (Zwieniecki *et al.*, 2001). The uptake of the contents of cells that are destroyed during girdling could potentially raise the ion concentration in the xylem sap (Windt *et al.*, 2009), whereas stopping the lateral ion exchange between the xylem and phloem via parenchyma cells might lower it (Zwieniecki *et al.*, 2004). As an increase in ionic concentration in the xylem sap is known to reduce the xylem hydraulic resistance (Zwieniecki *et al.*, 2001), this might influence xylem sap flow substantially. In girdled tree branches, this effect has been shown to be of potential importance (Zwieniecki *et al.*, 2004), although other experiments showed no effects of girdling on xylem ion concentration (Sellin *et al.*, 2013). While these ionic effects could possibly explain the observed change in the spatial distribution of xylem volume flow in the secondary xylem ring after girdling (Figs 5, S2), this did not lead to a significant alteration of the total amount of xylem volume flow (Fig. 6).

Another possible source of error with the subtractive method results from the fact that phloem girdling ceases sugar import

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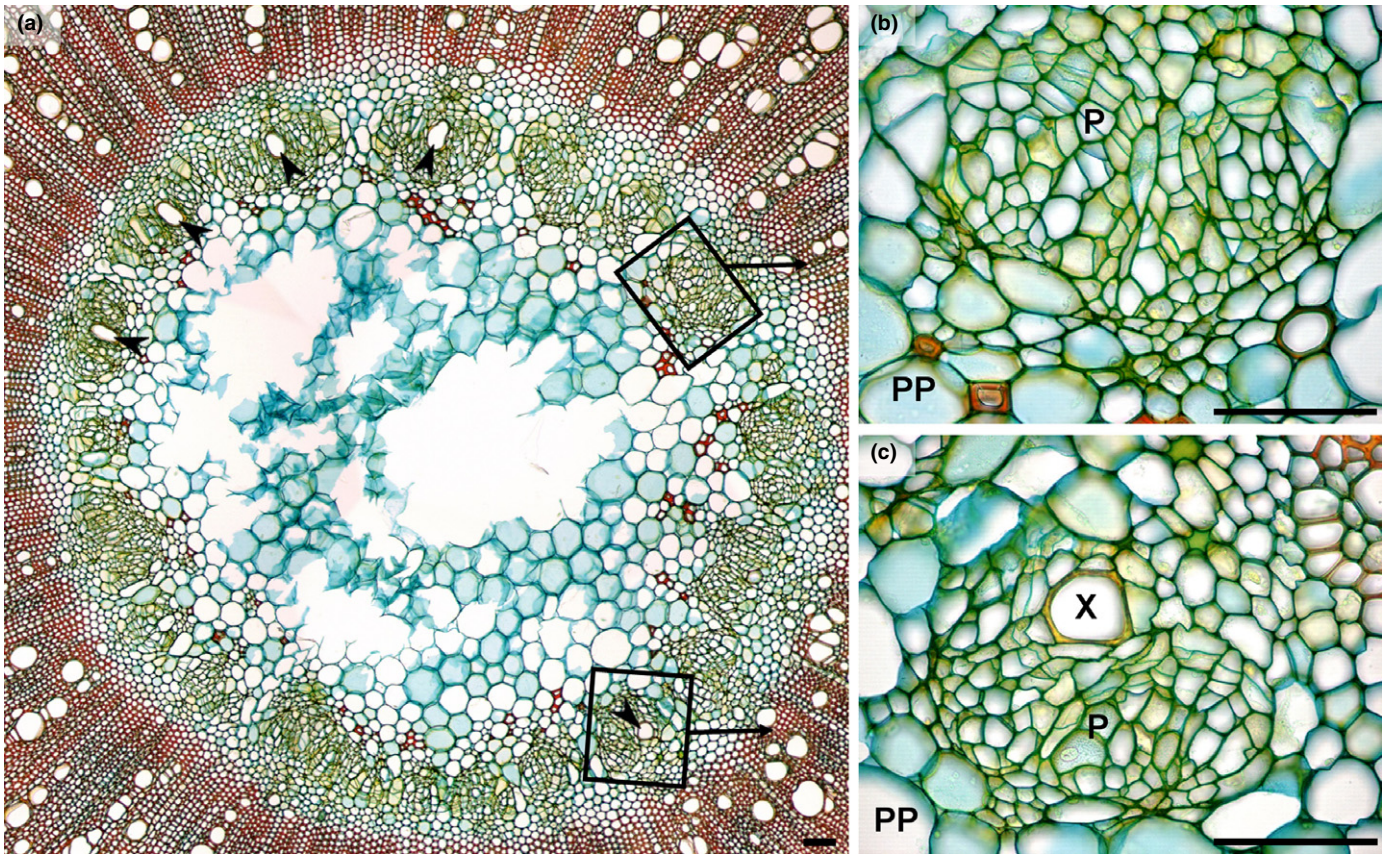

Fig. 4 (a) Overview of the anatomy of the vascular tissue in the perimedullary region of tomato truss 3 downstream from the location of girdling. (b, c) Detailed views of the phloem region (b) and a region with phloem enveloping a single xylem vessel (c). The central pith parenchyma is damaged by blade vibration during sectioning. The arrowheads indicate all single xylem vessels in the perimedullary region. X, xylem; P, phloem; PP, pith parenchyma. Bars, 100 μm.

into the fruit. If the xylem influx continues but the carbohydrate influx does not, the fruit's osmotic potential will alter. This might lead, in turn, to a decrease in the water potential gradient between fruit and stem, thus lowering the xylem influx (Fishman *et al.*, 2001; Hanssens *et al.*, 2015). The systematic relative error in xylem influx induced by this water potential difference is, however, low, as modelled for peach by Fishman *et al.* (2001), except at moments when the fruit volume does not change. Furthermore, Guichard *et al.* (2005) observed no change in osmotic potential over a time frame of 12 h after girdling in tomato, from which they concluded that xylem influx was not altered during this period. Their conclusion is not only supported by our findings, but, stronger still, we did not observe a decline in xylem influx for > 3 d after girdling (Fig. 7).

Xylem in the perimedullary region

Before girdling, the vascular tissue in the perimedullary region (Fig. 4) contributed between 17% and 29% to the total volume flow (Fig. 6). This tissue consists of phloem bundles (Fig. 4b), sometimes enclosing a single xylem vessel (Fig. 4c), as well as a small amount of parenchyma tissue in between the phloem bundles. Owing to the high cellular density, the limited resolution of the MRI flow imaging and the fact that both vascular tissues here conduct flow in the same direction, it was not possible to discriminate between xylem and phloem flow in this region. However, phloem tissue is much more prominent in this region (Fig. 4), suggesting that it may be the dominant contributor to the volume flow in the perimedullary region.

Nonetheless, we must take into account the fact that xylem flow in this region might not be entirely negligible. As girdling affects the entire perimedullary region, including the functioning of xylem vessels, this might result in an error when estimating xylem influx based on girdled peduncles. To assess the possible extent of this error, we took inventory of the number and size of the xylem vessels in the perimedullary region, and compared them with the number of xylem vessels in the secondary xylem ring of a comparable or larger size (i.e. secondary xylem vessels smaller than those in the perimedullary region were not counted). This comparison showed that the number of xylem vessels in the perimedullary region accounted for only 3.4 ± 0.9% of the total number of similarly sized or larger xylem vessels, and represented only 2.8 ± 0.8% of the total xylem area. We thus argue that the contribution of the xylem vessels in the perimedullary region to the total xylem volume flow must be small.

It is important to note that our trusses were 24–28 d after anthesis at the time of measurement, and thus were at a stage

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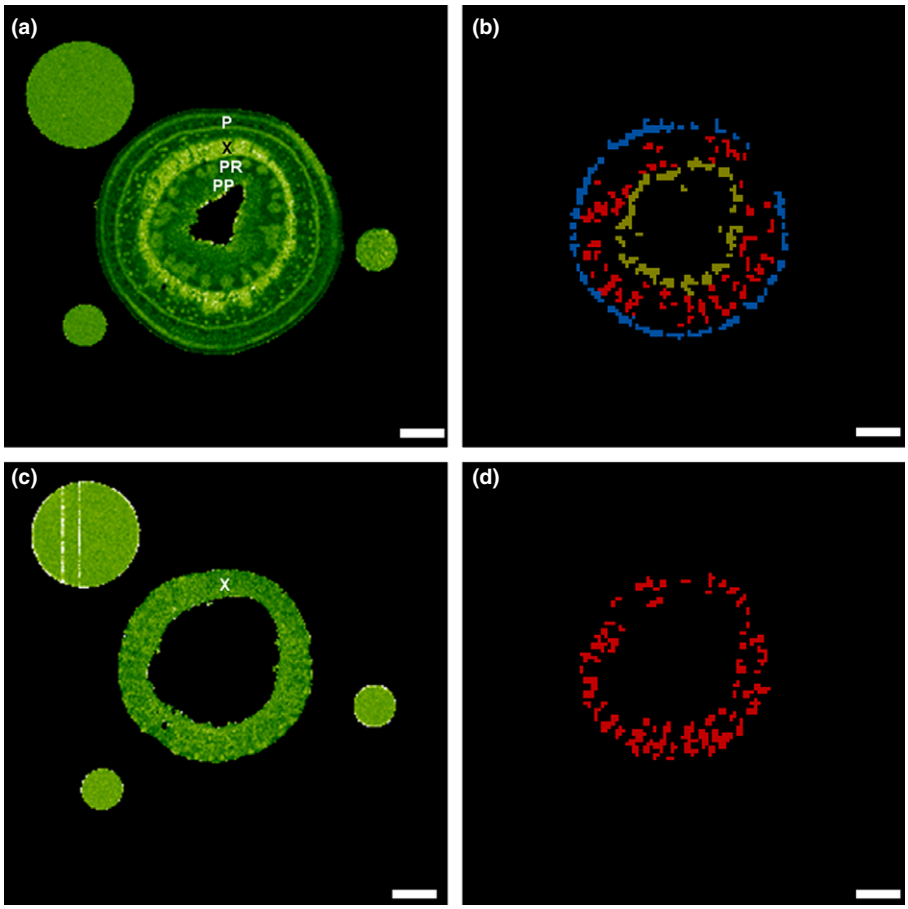

Fig. 5 Flow masks of water flow in the peduncle of tomato truss 4 before (a, b) and after heat girdling (c, d). The magnetic resonance images in the left columns represent the amount of stationary water and serve as an anatomical reference. The right columns represent the flow masks, marking the pixels that were found to contain flowing water in the xylem (red), phloem (blue) and perimedullary region (green). Please note that the flow masks only indicate the presence of vertically moving water, and do not indicate velocity or direction. P, phloem; X, xylem; PR, perimedullary region as a part of the pith parenchyma (PP). Bars, 1 mm.

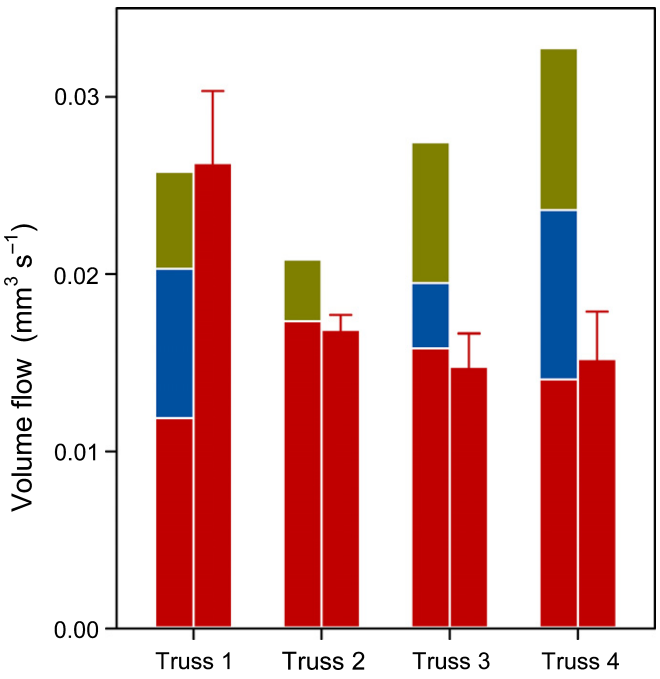

Fig. 6 The average total volume flow in xylem (red), phloem (blue) and the perimedullary region (green) of the tomato peduncle before (left bars) and after (right bars, ± SE) girdling, measured by magnetic resonance imaging velocimetry. SE values were calculated on subsequent measurements: $n = 2$ (trusses 1 and 2); $n = 7$ (truss 3); $n = 11$ (truss 4).

where the perimedullary region can still be assumed to contribute to the influx into the truss. Windt *et al.* (2009) observed that the perimedullary region only contributed notably to this influx up until the end of the fourth week of truss development. This means that, even if xylem contribution in the perimedullary region were more significant in younger tomato trusses, it would become negligible in older trusses.

Girdling: yea or nay?

This study is the first to experimentally assess the effects of heat girdling on xylem and phloem functionality, both qualitatively and quantitatively. Our results showed that, while all living tissues were destroyed, the secondary xylem remained not only physically, but also functionally, intact. Furthermore, despite the fact that volume flow patterns were slightly altered by heat girdling (Fig. S2), no significant changes in the amount of volume flow were observed in all but one peduncle.

However, some caveats should be taken into consideration. The first issue arises when measuring young peduncles: the fact that isolated xylem vessels in the perimedullary region do not remain functional after girdling induces errors the extent of which will be proportional to the amount of xylem volume flow in this region, which we were unable to quantify exactly. However, we argue that these errors are most likely small, and will become negligible as the truss matures.

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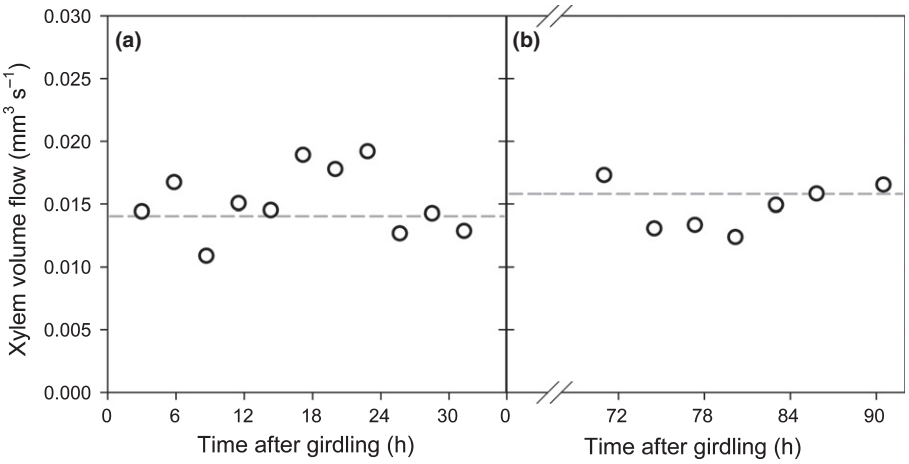

Fig. 7 The time course of the influx (volume flow) of water into the tomato truss, before and after girdling in truss 4 (a) and truss 3 (b). The grey dashed line represents the volume flow before girdling as a reference.

Furthermore, as heat girdling is destructive to living tissues, which is its purpose, it provides an indirect evaluation of phloem water flow rather than a direct *in vivo* measurement. This might lead to errors when experimental conditions before and after heat girdling are not identical. A clear example of this is truss 1, where an unintentional drought before heat girdling was thought to have led to a large discrepancy in xylem volume flow between the pre- and post-girdling measurements. If in such cases postgirdling measurements were used as an estimate for pre-girdling xylem contribution, large errors would arise.

As a result of these drawbacks related to indirect measurements, it is clear that the need for nondestructive, *in vivo* alternatives such as MRI or PET imaging to directly quantify xylem and phloem contribution remains. However, while these imaging techniques are powerful tools for measuring water and carbon fluxes (Hubeau & Steppe, 2015), even these currently lack the resolution to discriminate between xylem and phloem flow in the perimedullary region. While quantitative neutron imaging might overcome these resolution challenges, this technique is currently limited to measurements on small leaves and not applicable in the field (Defraeye *et al.*, 2014). Therefore, we argue that heat girdling currently remains the only practical technique to quantify xylem and phloem flow to the fruit in practical applications.

Here, we have refuted an array of existing concerns with this technique. We showed that heat girdling does not cause physical damage to the xylem, as both secondary xylem and singular xylem vessels in the perimedullary region remain intact (although the latter lose functionality); heat girdling does not cause embolisms of the secondary xylem vessels, either directly or in the longer term, and although xylem flow patterns can change slightly, the total volume flow through the secondary xylem is not significantly altered.

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Author contributions

B.A.E.V.d.W. and K.S. designed the experiment, B.A.E.V.d.W. and C.W.W. performed the experiment, C.W.W. processed the MRI data, O.L. performed the anatomical analysis, B.A.E.V.d.W., C.W.W. and K.S. interpreted the data, B.A.E.V.d.W. and C.W.W. wrote the manuscript, and all authors reviewed and commented on the manuscript.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

Fig. S1 Anatomy of the tomato peduncle at the location of girdling of truss 1, truss 2, truss 3 and truss 4.

Fig. S2 Volume flow maps of the peduncle before and after heat girdling for all tomato peduncles.

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