Sensing embolism in xylem vessels: the role of sucrose as a trigger for refilling

Sensing embolism in xylem vessels: the role of sucrose as a trigger for refilling

Short running title: **Sensing embolism in poplar vessels**

Francesca Secchi and Maciej A. Zwieniecki

Arnold Arboretum of Harvard University, Cambridge, MA, USA

Corresponding author: Francesca Secchi,
Harvard University, BioLabs 3114, 16 Dinivity Ave, Cambridge, MA 02138 USA
Email: fsecchi@oeb.harvard.edu

Abstract

Refilling of embolized vessels requires a source of water and the release of energy stored in xylem parenchyma cells. Past evidence suggests that embolism presence can trigger a biological response that is switched off upon successful vessel refilling. Since embolism formation is a purely physical process and most biological triggers rely on chemical sensors, we hypothesized that accumulation of osmotic compounds in walls of embolized vessels are involved in the embolism sensing mechanism. Analysis of *Populus trichocarpa*'s response to infiltration of sucrose, monosaccharides, polyethylene glycol and potassium chloride into the xylem revealed that only presence of sucrose resulted in a simultaneous physiological and molecular response similar to that induced by embolism. This response included reduction of the starch pool in xylem parenchyma cells and significant correlation of gene expression from aquaporins, amylases and sugar transporter families. The work provides evidence of the ability of plants to sense embolism and suggests that sucrose concentration is the stimulus that allows plants to trigger a biological response to embolism.

Keyword index: xylem, embolism, refilling, sucrose, signaling

Introduction

Embolism appears an inevitable part of everyday life for many vascular plants (Nardini & Salleo, 2000, Tyree & Sperry, 1988, Tyree & Sperry, 1989). At any given moment, even in non-stressed plants, there is a pool of embolized conduits that effectively reduce a plant's capacity to transport water (Domec, Scholz, Bucci *et al.*, 2006, Zwieniecki & Holbrook, 1998). The size of this conduit pool is highly variable and seems related to plant water potential (Tyree & Zimmermann, 2002). The temporal dynamics of xylem transport capacity suggests that many plant species are capable of removing air from embolized vessels even while actively transpiring and experiencing negative pressures in the bulk of apoplastic water (Nardini, Ramani, Gortan *et al.*, 2008, Salleo, Lo Gullo, De Paoli *et al.*, 1996). However, not all species appear able to repair dysfunctional conduits, and in many cases the recovery requires specific environmental conditions such as reduced transpiration rates or partial recovery from water stress (Hacke & Sperry, 2003). The notion of refilling, although still controversial, has become more of an accepted paradigm for understanding how the plant vascular system functions. Multiple studies have pointed out that the refilling process requires both a source of energy to overcome existing energy gradients acting against it, and a source of water to fill empty conduits (De Boer & Volkov, 2003, Holbrook & Zwieniecki, 1999, Tyree, Salleo, Nardini *et al.*, 1999). These two elements (energy and water) are vital for plant survival and are often present in limited quantities. Thus we can expect that their usage in the refilling process is optimized to avoid unnecessary losses (Zwieniecki & Holbrook, 2009).

Embolism is a result of cavitation occurring in the water column within the lumen of xylem vessels or tracheids. Cavitation is considered a spatially and temporally stochastic process related to the degree of tension and xylem physical properties (Holbrook & Zwieniecki, 1999, Tyree & Zimmermann, 2002). Thus, its temporal and spatial occurrence is unpredictable and the physiological response initiating refilling is likely not permanently associated with a specific part of the stem or limited to one period of a day. There are two potential solutions for plants to deal with the stochasticity of embolism. The first option requires that living cells adjacent to vessels always provide a continuous stream of energy (osmoticum) and water that could force refilling when embolism occurs. This option does not require a sensing mechanism, but is energetically wasteful. The second option assumes that the refilling process is triggered only when and where embolism occurs; this option results in energy conservation and thus might represent a preferred trait.

To discuss a viable hypothesis for a trigger mechanism that can turn the refilling process 'on' and 'off', we need a better understanding of the physical changes that accompany embolism formation and subsequent return of vessels to their functional state. As stated before, embolism is preceded by cavitation. In most cases, cavitation is an expansion of a vapor/gas void within a liquid phase when a threshold of liquid curvature (meniscus related force) cannot oppose the expansive pull of tension (Brennen, 1995). This initial expansion generates a pressure wave that forces the collapse of micro-bubbles near the front of the expanding embolus (Brennen, 1995) and produces an ultrasonic emission that spreads across the plant tissue. This initial fast embolism expansion is followed by a slow phase, limited by the rate at which water can leave the vessel (i.e.

pressure gradient and hydraulic resistance of bordered pits and walls). At the end of embolism formation, the walls of the vessel remain wet with water potentials similar to that of the vessel at the time of cavitation. Thus living cells adjacent to vessels experience conditions similar to the pre-embolism state thus leaving an open question as to what triggers the refilling mechanism.

Recently it was suggested that ultrasonic events could be responsible for triggering the refilling process (Salleo, Trifilo' & Lo Gullo, 2008). While generally possible, the mechano-sensing properties of wood parenchyma cells would have to be extremely sensitive to translate a millisecond-long acoustic event into biological activity that could last for hours. Zwieniecki and Holbrook (2009) suggested an alternative mechanism that relies on the cells' ability to detect changes in osmotic potential or concentrations of specific molecular compounds in vessels walls. In brief, this mechanism requires a small steady release/leak of osmoticum/molecules from parenchyma cells, which diffuse through the cellular walls to the vessel lumen. When the vessel is functional, the evaporation stream carries this osmoticum away, maintaining a low concentration in the walls of the parenchyma/vessel complex. In the case of embolism, osmoticum is not carried away and it accumulates in the vessel walls. This increased concentration provides an 'on' signal, triggering the refilling process and active pumping of solute out of the cells, which provides a driving force for water flow into the vessel. The refilling process will continue until the concentration in the vessel wall drops to pre-embolism levels due to restoration of the transpirational stream. This dilution provides an 'off' switch for the refilling process (Zwieniecki & Holbrook, 2009). Although theoretically plausible, this proposal has not been tested experimentally.

In this manuscript, we test the hypothesis that changes in xylem sap molecular composition can trigger a response similar to that observed following embolism formation.

Materials and Methods

Plant materials and growth conditions

Populus trichocarpa cuttings were rooted in aerated hydroponics containers (6.5 L) filled with modified Hoagland solution (pH 6.50-7.00; 795 µM KNO_3, 603 µM $Ca(NO_3)$ 270 µM $MgSO_4$ and 190 µM KH_2PO_4; micronutrients: 40.5 µM Fe(III)-EDTA, 20 µM H_3BO_4, 2 µM $MnSO_4$, 0.085 µM $ZnSO_4$, 0.15 µM $CuSO_4$ and 0.25 µM Na_2MoO_4) and located in a controlled growth chamber (24/20°C day/night, 12/12 light/dark, 70% humidity with 500 µmol m^{-2} s^{-1} PAR). After two to three weeks, the plants were transferred to 42 L boxes (12 plants per box) and allowed to grow for an additional three to four months. The nutrient solution was replaced weekly. Plants were approximately 70 cm tall at the outset of the experiments. The average stem diameter was 6.5 ± 0.84 mm at a distance of 10 cm from root collar.

Artificial induction of embolism

A group of 36 poplars was used to determine recovery from artificially induced embolism. Eighteen plants were embolized while another eighteen plants were used as controls. The injection treatment followed the procedure described previously by Secchi & Zwieniecki (2010). In brief, an incision was made in the stem using 1.0 mm drill bit

and a small diameter tube (0.98 mm; PEEK - polyether ether ketone) was sealed to the incision using a custom-built compression fitting system that allowed for direct pressurization of the xylem. An air pressure of ~1 MPa was applied to the stems for 60 seconds after which time the pressure was released and the holder removed. Stem percent loss of conductivity (PLC) was determined immediately, half an hour and two hours following the injection on 3 cm long sections located approximately 10 cm from the incision. The 10 cm distance was determined to be more than the average vessel length in studied plants and it was chosen to avoid embolism formation due to incision and vessel damage. The wood from the remaining distal portion of the stem was collected for the starch content determination and gene expression analysis (for details see below). To prevent contamination of the wood by bark or phloem, the bark was peeled and the outside xylem was scraped with a scalpel. Samples were immediately frozen in liquid nitrogen and kept at - 80°C until analyzed. Control plants were treated exactly as injected plants including incision and chamber placement, though no pressure was applied.

Osmotic stress treatments

Experiments were conducted on 72 poplars. Plants were divided into two groups. The first group was used to test the effect of osmotic stresses and high sugar concentrations on gene expression and wood starch content after 2h of exposure to each treatment. The second group was used to follow temporal changes in starch content and gene expression after 0.5, 2 and 4 hours of exposure to treatments. The thirty-six poplar plants belonging to the first group were further divided into 5 subgroups. Twelve plants were kept as controls. The remaining 24 plants were randomly assigned to four different

147 treatments (6 plants per treatment) with osmotic stress induced by adding 1MPa of: (1)
148 KCl (30 g L^{-1}), (2) polyethylene glycol (PEG200; 82 g L^{-1}), (3) sucrose (137 g L^{-1}), and
149 (4) a mixture fructose and glucose (37 g L^{-1}). The tested substances were added to the
150 modified Hoagland solutions and the mixture was aerated for at least two hours before, as
151 we previously determined that lack of pre-aeration leads to sudden loss of turgor. To
152 assure uptake of chemicals, we shaved approximately 50% of the root tips from the root
153 system using razor blades before we immersed the plants in the modified solutions. Test
154 with diluted Safranin solution demonstrated that dye reached the leaves within a few
155 minutes following the root shaving procedure. We collected wood samples for molecular
156 and sugar analysis at the beginning of the treatments (six control plants) and after 2h of
157 exposure to each treatment (control and treated plants). During the experiment, plants
158 were kept in typical 'day' condition of growth chamber.
159
160 *Temporal response to sugar treatments*
161 To follow the temporal response to the sugar treatments, another 36 poplars with
162 shaved roots were immersed in aerated modified Hoagland nutrient medium (control
163 plants) or in an aerated medium supplemented with either sucrose or a glucose- fructose
164 mixture (concentration pressure equivalent of 1.0 MPa). The preparations of solutions
165 and plants were the same as described above. After two hours, the root systems of six
166 plants in the sugar treatments were rinsed with distilled water and shifted to a regular
167 hydroponic medium where they were kept for an additional two hours (recovery plants).
168 Wood samples for molecular and sugar analyses were collected immediately after plant
169 preparation (control plants) and after 0.5, 2 and 4 hours of exposure to treatments.

Measurements of stem water potential and stem hydraulic conductivity

Stem water potential was measured for each plant using equilibrated non-transpiring (bagged) leaves. Mature leaves were covered with aluminum foil and placed in a humidified plastic bag for 15 minutes prior to excision and measurement. After excision, leaves were allowed to equilibrate for more then 10 minutes before water potential was measured using a Scholander-type pressure chamber (Soil Moisture Equipment Corp., Santa Barbara, CA, USA).

Following the determination of stem water potential, stem hydraulic conductivity was measured using a standard approach described previously (Secchi & Zwieniecki, 2010). In brief, sections of stems (~ 3 cm long) were cut under water directly from intact plants. The initial hydraulic conductance (k_i) of each stem segment was measured gravimetrically by determining the flow rate of filtered 10 mM KCl solution through the stem section from a water source located on a balance (Sartorius ±0.01 mg) and connected to the stem by a plastic tube. The stem was submerged in a water bath with a water level ~10cm below that on the balance. After a steady flow rate was reached (within a few minutes), the tube connecting the stem to the balance was closed, and a bypass tube was used to push water across the segment under ~ 2 bars of pressure for approximately 20 seconds to remove embolism. Stem conductance was then re-measured to find maximum conductance (k_{max}). The percent loss of conductance (PLC) was calculated as:

$$PLC = 100 * (k_{max}-k_i)/k_{max}$$

This approach relies on the previously tested assumptions (Melcher, Zwieniecki & Holbrook, 2003) that most vessels in short segments are open and that a low water head does not push emboli from these vessels. In such system, embolism removal is easy since the embolism does not have to be dissolved back into water but is simply pushed out of the vessel with a 2 bar pressure.

Database sequence search and primer design

Amylase and sucrose transporter sequence genes were identified in the *P. trichocarpa* annotated genome version 2.0 (http://www.phytozome.net/poplar.php) using one *A. thaliana* amylase protein sequence (AtAMY2) and one sucrose transport protein sequence (AtSUT2) as BLAST query sequences. Thus, the identified sequences were chosen as queries to identify additional *Populus* homologs. The gene name, annotation name and the peptide homology with *Arabidopsis thaliana* sequences are reported in Table 1. Primer 3 program (http://frodo.wi.mit.edu/primer3/) was used to design specific primers for amylase and sucrose transporters sequences, while the primers already listed in Secchi et al. (Secchi, MacIver, Zeidel *et al.*, 2009) were used to study the aquaporin gene expression (see Supplemental Table S1).

Semi-quantitative Real-Time PCR

Collected wood samples were grounded to a fine powder in liquid nitrogen and 500 mg of powder from two different trees were mixed together to obtain one biological replicate. Total RNA was isolated according to Chang et al.'s protocol (Chang, Puryear & Cairney, 1993) and contaminant genomic DNA was removed from the samples by

digestion with RNase-free DNase I (Fermentas), following the manufacturer's instructions. cDNA was synthesized using SuperScript II Reverse Transcriptase (Invitrogen) according to the supplier's instructions, using oligo-(dT)12–18 (Fermentas) as a primer.

Gene transcript abundance was quantified with SYBR Green JumpStart *Taq* ReadyMix (Sigma) on an Mx3000PTM PCR system (Stratagene). Thermocycler conditions for all real-time analyses were 95 °C for 5 min, followed by 40 cycles of 95 °C for 30 s, 58°C for 1 min and 72 °C for 30 s. Data were analyzed using Mx3000PTM Real-Time PCR system software (Stratagene) and the values were normalized to transcript levels of the ubiquitin gene. PtBMY2, PtSUT1.2 and PtBMY4 genes were not included in this analysis since the first two genes were expressed at very low levels in wood (Supplemental Data, Figure S1) and PtBMY4 was not detected in the tissue.
Real time PCR was carried out using three biological replicates per collection time and treatment. Three technical replicates (repeats of the cDNA quantification) were performed for each of the three biological replicates.

Sucrose and starch analysis

Collected wood samples were ground to a fine powder with a mortar and pestle in liquid nitrogen and subjected to starch and sucrose assays. The samples subjected to starch determination were extracted with 80% ethanol for 10 min at 85°C with constant shaking and then centrifuged at 10,000g for 20 min. The resulting pellet was washed three times with 80% (v/v) ethanol and analyzed using a starch assay kit (STA-20, Sigma-Aldrich, St. Louis). The pellet was then immersed in boiling water for 5 min, and digested

with α-amylase and α-amyloglucosidase according to the manufacturer's protocol. The starch content was represented by the amount of released glucose, which was assayed colorimetrically using a glucose oxidase-mediated method (STA-20, Sigma-Aldrich, St. Louis). Absorbance was read at 540 nm. Three technical replicates of 90-100 mg were assayed for each plant.

For glucose assays, three replicates of wood tissue (90-100 mg) were extracted with deionized water for 15 minutes at 70°C and then centrifuged at 10,000*g* for 20 min. A fraction of the supernatant phase containing soluble sugar extracts was determined using the Glucose Assay kit (GAGO-20, Sigma-Aldrich, St. Louis). Glucose (free glucose) was assayed colorimetrically using glucose oxidase and the relative absorbance was read at 540 nm. Another fraction of the same supernatant was digested with 15 units of invertase (Sigma) and total glucose level was measured. The sucrose level was calculated as total glucose minus free glucose. In all cases starch and sucrose concentrations were expressed per fresh woody biomass, and included both cellular and apoplastic sugar content.

Results

Artificial induction of embolism

Artificial induction of embolism in stems of *P. trichocarpa* resulted in moderate to severe loss of stem hydraulic capacity as expressed as percent loss of conductivity (PLC; see methods for the PLC determination procedure). A PLC of more than 70% was recorded following air injection. At the same time, PLC in control plants remained below 20%. We observed a small recovery from embolism within half an hour after inducing

embolism and almost full recovery after 2-hours (Fig 1A). Leaf water potential of both control and air-injected plants was unaffected or was even less negative over the progression of experiment as root tips of the plants were cut and root resistance most likely reduced (Fig. 1B). Analysis of starch content in stems of control plants showed a relatively constant level over the duration of the experiment. However, in air-injected plants, we observed a small, but significant, drop in starch content in the woody part of the stem (Fig 1C), from 11.9 to 10.4 [mg/g] ($df=8$ t-value=2.43 and $p=0.041$) after two hours. This drop of approximately 1.5 mg/g of starch was met by a small, but significant, increase in sucrose content in stems of embolized plants from 2.1 to 2.8 mg/g ($df=9$ t-value=3.33 $p= 0.009$; Fig. 1D).

This starch related metabolic response was met with a small but significant up-regulation of two aquaporins PtPIP1.1 and PtPIP1.3 genes within half an hour following the air injection treatment (Fig. 2A). Expression of the remaining aquaporin genes did not show any significant difference between control and treated plants within that time-frame. Sucrose transporter genes in the xylem tissue showed significant up-regulation of PtSUC2.1 and PtSUT2b during the first half hour following treatment (Fig. 2A). In general, two hours following air injection, expression of the tested aquaporin genes were slightly up-regulated while a majority of the tested amylase and sucrose transporter genes were down regulated, with the exception of PtSUC2.1 (Fig. 2A).

Osmotic stress treatments

Of the four tested osmolite solutions (PEG, KCl, sucrose and fructose-glucose mixture), only the presence of sucrose in the xylem triggered a significant degradation of

starch, from 9.5 to 6.2 mg/g (df=14 t-value=2.20 and 2=0.045; Fig. 3). A small drop was also observed in the KCl treatment, from 9.5 to 7.6 mg/g, but was not statistically significant (df=10 t-value=1.16 and p=0.27). The other two treatments (PEG and monosaccharides) did not cause a starch concentration change. Analysis of gene expression in response to these different osmolite solutions treatments showed an interesting mosaic of responses. In general, genes from the PtPIP2 subfamily, responded with a strong and significant up-regulation of expression, with the exception of PtPIP2.2 gene (Fig. 2B). We also noted up-regulation of PtPIP1.1 and PtPIP1.3 genes. Amylase and sucrose transporter genes responded differently to osmotic (PEG and KCl) and sugar treatments. During osmotic-stress treatments, the genes were either down-regulated or did not change, with the exception of PtBMY1a (strongly up-regulated), (Fig. 2B). Sugar treatments resulted in up-regulation of sucrose transporters and a mixed response of amylases where some were strongly up-regulated (PtBMY1a and Pt BMY3) and some down-regulated (PtAMY2, PtAMY3 and PtBMY5; Fig. 2B).

Temporal response to sugar treatments

Sucrose treatment did not cause any significant change in the starch content over the half hour treatment (10.5 to 9.4 [mg/g] with df=7 t-value=0.49 and p=0.64; Fig. 4). However, the two-hour drop described before persisted for four hours with a drop in starch content from 11 to 7.4 [mg/g] (df=6 t-value=3.84 and p=0.008). Application of the fructose and glucose mixture did not affect starch content in the stem over half an hour (control and treated plants respectively 11.8 to 9.4 [mg/g] with df=8 t-value=1.30 and p=0.22) or prolonged exposure for four hours (12.6 to 11 [mg/g] with df=7 t-value=0.52

and p=0.61). The recovery treatment (shifting plants from sugar solutions to regular hydroponic medium) resulted in a slight, although not significant, increase in starch content in the fructose and glucose treated plants, and increased variation in the starch content in sucrose treated plants (Fig. 4). Within half an hour of the sugar treatment, there was a significant change in the expression pattern of sucrose transporters (Fig. 2C and 2D). In addition, the sucrose application corresponded with significant changes in the expression of a few amylase genes. This initial (half an hour) response was later (2 and 4 hour following the treatment) supplemented with very strong up-regulation of PtPIP genes (including 1.1, 1.3, 2.3, 2.4 and 2.6; Fig. 2C). Fructose and glucose application had similar effect but it did not cause any additional long-term changes in amylase expression with the exception of the PtBMY1a (Fig. 2D).

Comparative analysis of gene expression among treatments

Comparative analysis of gene expression in air-injected plants, and those treated with different osmolites, showed important co-expression patterns. In general, aquaporin genes were up-regulated in both sets of experiments (Fig. 5). However, in PEG and KCL treatments, amylases and sugar transporters genes showed little similarity in expression level with air-injected plants; no significant correlations existed between expression levels (PEG: n=20 r^2=0.09 and p=0.19; KCl: n=20 r^2=0.19 and p=0.054) as tested on logarithm of expression fold change in relation to the control. The correlation of gene expression, however, was significant between sugar treatments and air injection (sucrose: n=20 r^2=0.21 and p=0.045; fructose and glucose: n=20 r^2=0.28 and p=0.017; Fig. 5). The presence of correlation between expression patterns of tested genes for air injected and

sugar treated plants suggests that, despite obvious treatment differences in these two experiments, they still triggered similar expression pathways, and therefore may share the same signaling cascades. These correlations, between expression levels of air-injected and sugar-treated plants, however, did not appear until 2-hours after treatment (Supplemental Data, Figure S2). The lack of correlation half an hour after treatment might by due to overall low logarithm fold change at this point in time.

Discussion

Starch metabolism is considered to be at the forefront of plant response to embolism (Bucci, Scholz, Goldstein *et al.*, 2003, Regier, Streb, Cocozza *et al.*, 2009, Salleo, Lo Gullo, Trifilo *et al.*, 2004). It has been suggested that starch provides the source of energy required to overcome the energy gradients opposing the refilling process (Salleo, Trifilo & Lo Gullo, 2006, Zwieniecki & Holbrook, 2009). In this study, we found an association between starch reduction and the refilling process in *P. trichocarpa* plants, as was formerly shown for *L. nobilis* plants (Salleo, Trifilo', Esposito *et al.*, 2009). Specifically, we observed a significant drop in starch concentration in the xylem of plants refilling after an artificially induced embolism. This drop was associated with a small increase in sucrose content that was similar in molecular weight to reduction observed in starch. This result further confirms the role of starch in the refilling process, and suggests that starch content can serve as an indicator for refilling process. It also suggests that in the case of plants with very low starch content, such as severely stressed plants, refilling might be delayed or absent (Secchi & Zwieniecki, 2010).

352 The fact that the presence of embolism induces up-regulation of starch
353 metabolism genes suggests that plants can detect embolism and translate a purely
354 physical process into a biological response. As described in the introduction we believe
355 that the formation of embolism is not associated with any extreme (from the plant's point
356 of view) physical event – which is particularly true for the artificial embolism induction
357 used in our experiment. We pushed water out of vessels with a pressure differential of
358 only 1.0 MPa. Such pressures/tensions are frequently experienced by the plant xylem in
359 nature. Following the pressure treatment, approximately 30% of conduits were still
360 functional and the remaining flow capacity was adequate to maintain unchanged overall
361 plant water status as determined by leaf water potential, although it is possible that a drop
362 in stomatal conductance was present. In addition, such artificial induction of embolism
363 removes the typical physiological triggers related to drought stress, like osmotic
364 adjustments, continuous supersonic acoustic emissions associated with embolism or
365 changes in phloem function that might result from axial water potential alterations. Thus,
366 the presented results provide support for the proposal that response to embolism presence
367 is triggered by the disruption of vessel transport and possibly associated with changes in
368 the balance of osmotic concentrations between parenchyma cells and adjacent vessels
369 (Zwieniecki & Holbrook, 2009; Fig. 6). Interestingly, when we substituted artificial
370 induction of embolism for direct application of osmotic solutions to xylem, the presence
371 of sucrose in the xylem induced a drop in starch concentration that matched that of the
372 induced embolism. Applications of other compounds like polyethylene glycol (PEG),
373 KCl salt or a mixture of monosaccharides did not cause any significant change in starch
374 content despite being applied at the same osmotic pressure as sucrose (1.0 MPa). This

finding suggests that the presence of sucrose, but not the other tested osmotically active substances, induces a physiological response similar to that which occurs after embolism induction.

Furthermore both embolism and apoplastic sucrose similarly influenced the expression of genes assumed to participate in embolism refilling. Specifically, the sucrose transporter gene (PtSUC2.1) was significantly up-regulated in response to both embolism and sucrose treatments (2 hours). It is interesting that this gene shares an high homology (68.96% identical amino acids) with *Juglans regia* L. sucrose transporter gene (JrSUT1), as this gene was expressed mainly in vessel-associated cells and its up-regulation was related to the ability of walnut trees to repair xylem embolism after freeze-thaw induction (Decourteix, Alves, Brunel *et al.*, 2006). It was also shown that a rise in sucrose concentration in walnut trees, related to freeze-thaw cycles, resulted in an increased sucrose apoplastic concentration, reduced amount of starch and up-regulation of water channel genes from the PIP2 subfamily (Sakr, Alves, Morillon *et al.*, 2003). A similar response was observed in our study following the artificial induction of embolism. We noted a drop in starch concentration, increased sucrose concentration and up-regulation of aquaporins from PIP2 subfamily two hours post treatment. This response of the plant to embolism was matched by the response to sucrose treatment, congruent with the notion that sucrose is an essential part of the embolism sensing mechanism. Other applied osmotica influenced the expression of some genes in similar manner, but only the treatment with sucrose influenced the starch content, underlining its involvement in plant response to embolism formation.

Can sucrose be involved in embolism detection? There is no doubt that sugars are involved in many signaling processes in plants (Guptka & Narinder, 2005, Loreti, De Bellis, Alpi *et al.*, 2001a). Sucrose is the major compound transported in the plant and present across all tissues, both in apoplastic and symplastic compartments; apoplastic sucrose is sensed by membrane sucrose transporters (Guptka & Narinder, 2005, Loreti, De Bellis, Alpi *et al.*, 2001b). Thus it seems parsimonious that if there are embolism-associated changes in apoplastic sucrose concentration, such information can be used by adjacent cells to trigger a specific response. While it was not our goal to show how sucrose reaches vessels, we speculate that the phloem transport system continuously leaks sucrose (van Bel, 2003), which then diffuses across the stem to the transpiration stream, where low levels of carbohydrates have been detected (Johnson, Tyree & Dixon, 1987). When the large volume of water in vessels disappears due to embolism, an accumulation of sucrose in the apoplast (vessel walls) is clearly plausible. Since wall volume is small in comparison to vessel lumen, the accumulation of the sucrose or other osmoticum could be very rapid and capable of triggering specific signaling paths responsible for the refilling. Obviously, such sucrose accumulation also changes the chemical water potential. Thus some of the plant response reported here could be due to a non-specific response to changes in water potential i.e. similar in part to the responses triggered by PEG, KCl or water stress.

It was noted that changes in gene expression following induction of embolism were less pronounced than in the sucrose treatment. This might be the result of experimental design, because response to embolism is spatially limited to empty vessels and a few living cells surrounding them, while the osmotic treatment affects all vessels

and all parenchyma cells in the stem, magnifying the level of response. It is also possible that solute osmotic pressure (~1 MPa) was an exaggeration of the real accumulation of sucrose in the walls of the vessels following embolism. Although, considering the need for the osmotic energy to refill embolized vessels, the maintaince of such concentration would be extremely important to generate the required energy gradients. Thus, we expect that there might be feed-forward control mechanism stimulating release of sucrose from cells if a threshold concentration value in vessel wall was reached. It is also important to note that changes in gene expression resulting from the embolism and osmotic treatments were significantly correlated for sugars and less so for the PEG or KCl treatments. This reinforces our proposal that both embolism and sucrose response share the same signaling pathways.

This work cannot fully resolve whether sucrose accumulation in vessel walls is the initial mechanism triggering a refilling response to embolism. The allure of this hypothesis is that it provides a mechanism for the cessation of the refilling response – that post refilling flow of water through the vessel lowers the sucrose concentration below the level that triggered the initial refilling response. Here we demonstrate that the expression levels of putative refilling-related genes (aquaporins, and sugar metabolism and transport genes) after air injection induced embolism are similar to those induced by sugar addition to the xylem, and that addition of other osmotica did not stimulate this expression response. In addition, we showed that embolism induction and sucrose treatments have similar responses in terms of starch content changes – a likely source of energy for the refilling process. Together, we feel that this evidence provides strong support for the role of sucrose in the mechanism of embolism sensing (Fig. 6).

Acknowledgements

The authors wish to thank Matthew Gilbert, Rebecca Neumann and Alicia Pou for help and support during the experimental work and preparation of the manuscript. Financial source: Mercer Fellowship in Plant Biology provided by Arnold Arboretum of Harvard University to Francesca Secchi and National Science Foundation Award #: IOS-0919729

References

Brennen C.E. (1995) *Cavitation and bubble dynamics* (vol. 44). Oxford University Press, New York.

Bucci S.J., Scholz F.G., Goldstein G., Meinzer F.C., Da L. & Sternberg S.L. (2003) Dynamic changes in hydraulic conductivity in petioles of two savanna tree species: factors and mechanisms contributing to the refilling of embolized vessels. *Plant, Cell and Environment*, **26**, 1633-1645.

Chang S., Puryear J. & Cairney J. (1993) A simple and efficient method for isolating RNA from pine tree. *Plant Molecular Biology Report*, **11**, 113-116.

De Boer A.H. & Volkov V. (2003) Logistics of water and salt transport through the plant: structure and functioning of the xylem. *Plant Cell and Environment*, **26**, 87-101.

Decourteix M., Alves G., Brunel N., Ameglio T., Guilliot A., Lemoine R., Petel G. & Sakr S. (2006) JrSUT, a putative xylem sucrose transporter, could mediate sucrose influx into xylem parenchyma cells and be upregulated by freeze–thaw

cycles over the autumn–winter period in walnut tree (*Juglans regia* L.). *Plant Cell and Environment*, **29**, 36-47.

Domec J.C., Scholz F.G., Bucci S.J., Meinzer F.C., Goldstein G. & Villalobos-Vega R. (2006) Diurnal and seasonal variation in root xylem embolism in neotropical savanna woody species: impact on stomatal control of plant water status. *Plant Cell and Environment*, **29**, 26-35.

Guptka A.K. & Narinder K. (2005) Sugar signalling and gene expression in relation to carbohydrate metabolism under abiotic stresses in plants. *J. Biosci.*, **30**, 761-776.

Hacke U.G. & Sperry J.S. (2003) Limits to xylem refilling under negative pressure in Laurus nobilis and Acer negundo. *Plant Cell and Environment*, **26**, 303-311.

Holbrook N.M. & Zwieniecki M.A. (1999) Xylem refilling under tension. Do we need a miracle? *Plant Physiology*, **120**, 7-10.

Johnson R., Tyree M. & Dixon M. (1987) A requirement for sucrose in xylem sap flow from dormant maple trees. *Plant Physiology*, **84**, 495-500.

Loreti E., De Bellis L., Alpi A. & Perata P. (2001a) Why and how do plant cells sense sugars? *Annals of Botany*, **88**, 803-812.

Loreti E., De Bellis L., Alpi A. & Perata P. (2001b) Why and how do plant cells sense sugars? . *Annals of Botany*, **88**, 803-812.

Melcher P.J., Zwieniecki M.A. & Holbrook N.M. (2003) Vulnerability of xylem vessels to cavitation in sugar maple. Scaling from individual vessels to whole branches. *Plant Physiology*, **131**, 1775-1780.

Nardini A., Ramani M., Gortan E. & Salleo S. (2008) Vein recovery from embolism occurs under negative pressure in leaves of sunflower (*Helianthus annuus*). *Physiologia plantarum*, **133**, 755-764.

Nardini A. & Salleo S. (2000) Limitation of stomatal conductance by hydraulic traits: sensing or preventing xylem cavitation? *Trees*, **15**, 14-24.

Regier N., Streb S., Cocozza C., Schaub M., Cherubini P., Zeeman S. & Frey B. (2009) Drought tolerance of two black poplar (*Populus nigra* L.) clones: contribution of carbohydrates and oxidative stress defence. *Plant Cell and Environment*, **32**, 1724-1736.

Sakr S., Alves G., Morillon R.L., Maurel K., Decourteix M., Guilliot A., Fleurat-Lessard P., Julien J.L. & Chrispeels M.J. (2003) Plasma membrane aquaporins are involved in winter embolism recovery in walnut tree. *Plant Physiology*, **133**, 630-641.

Salleo S., Lo Gullo M.A., De Paoli D. & Zippo M. (1996) Xylem recovery from cavitation-induced embolism in young plants of *Laurus nobilis*: a possible mechanism. *New Phytologist*, **132**, 47-56.

Salleo S., Lo Gullo M.A., Trifilo' P. & Nardini A. (2004) New evidence for a role of vessel-associated cells and phloem in the rapid xylem refilling of cavitated stems of Laurus nobilis L. *Plant, Cell and Environment*, **27**, 1065-1076.

Salleo S., Trifilo' P., Esposito S., Nardini A. & Lo Gullo M.A. (2009) Starch-to-sugar conversion in wood parenchyma of field-growing *Laurus nobilis* plants: a component of the signal pathway for embolism repair? *Functional Plant Biology*, **36**, 815-825.

509 Salleo S., Trifilo' P. & Lo Gullo M.A. (2008) Vessel wall vibrations: trigger for
510 embolism repair? *Functional Plant Biology*, **35**, 289-297.
511 Salleo S., Trifilo' P. & Lo Gullo M.A. (2006) Phloem as a possible major determinant of
512 rapid cavitation reversal in stems of *Laurus nobilis* (laurel). *Functional Plant*
513 *Biology* **33**, 1063-1074.
514 Secchi F., MacIver B., Zeidel M.L. & Zwieniecki M.A. (2009) Functional analysis of
515 putative genes encoding the PIP2 water channel subfamily in *Populus*
516 *trichocarpa*. *Tree Physiology*, **29**, 1467-1477.
517 Secchi F. & Zwieniecki M.A. (2010) Patterns of PIP gene expression in *Populus*
518 *trichocarpa* during recovery from xylem embolism suggest a major role for the
519 PIP1 aquaporin subfamily as moderators of refilling process. *Plant Cell and*
520 *Environment*, **33**, 1285-1297.
521 Tyree M.T., Salleo S., Nardini A., Lo Gullo M.A. & Mosca R. (1999) Refilling of
522 embolized vessels in young stems of Laurel. Do we need a new paradigm? *Plant*
523 *Physiology*, **120**, 11-21.
524 Tyree M.T. & Sperry J.S. (1988) Do woody plants operate near the point of catastrophic
525 xylem dysfunction caused by dynamic water stress? :answers from a model. *Plant*
526 *Physiol*, **88**, 574-780.
527 Tyree M.T. & Sperry J.S. (1989) Vulnerability of xylem to cavitation and embolism.
528 *Annual Reviews of Plant Physiology and Molecular Biology*, **40**, 19-38.
529 Tyree M.T. & Zimmermann M.H. (2002) *Xylem structure and the ascent of sap*. (2nd
530 ed.). Springer, Berlin.

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

Zwieniecki M. & Holbrook N. (2009) Confronting Maxwell's demon: biophysics of xylem embolism repair. *Trends in Plant Science*, **14**, 530-534.

Zwieniecki M.A. & Holbrook N.M. (1998) Diurnal variation in xylem hydraulic conductivity in white ash (*Fraxinus americana* L.), red maple (*Acer rubrum* L,) and red spruce (*Picea rubens* Sarg,). *Plant Cell and Environment*, **21**, 1173-1180.

Table 1: Gene name, Phytozome and JGI sources, sequences of primers used for quantitative Real Time PCR.

Gene name	Phytozome V 5.0 (locus name)	JGI V1.1 (ID)	JGI V1.1 (gene name)	Peptide Homologs + *A. thaliana* locus name	Forward primer (5'-3')	Reverse primer (5'-3')
PtAMY1	POPTR_0515s00220	644125	grail3.0033012902	80.9% AMY1 (AT4G25000)	GGAATTAAATGCCTGGCAGA	GGATTTCCTTTGCCATCAGA
PtAMY2	POPTR_0002s01570	798380	fgenesh4_pm.C_LG_II000069	90% AMY2 (AT1G76130)	TATGCCGTGTGGCAAAAATA	AAAGGGCATCACTCATCCAC
PtAMY3	POPTR_0010s10300	231366	gw1.X.6063.1	76.3% AMY3 (AT1G69830)	GTCATTACGAACCGCCAAGT	CAGGCGGACACTTTCTTGAT
PtBMY1a	POPTR_0008s17420	832848	estExt_fgenesh4_pm.C_LG_VIII0731	86.7% BAM1 (AT3G23920)	AGAGTCCTGACCGGTGTTTG	GGCTGCTGTTCCTGTGATTT
PtBMY1b	POPTR_0001s11000	179884	gw1.I.8484.1	55.7% BAM1 (AT3G23920)	TAAGGTCTTGACGGCGTCTT	ACCCACACCGTCTTGCTAAC
PtBMY2	POPTR_0003s10570	414016	gw1.III.1119.1	77.2% BMY2 (AT5G45300)	CCGACCCGTATTCTCACCTA	TACCTTTACCCGAGGCATTG
PtBMY3	POPTR_0008s20870	420524	gw1.VIII.1952.1	75.7% BMY3 (AT5G18670)	AGTCAAACACCCACATGCAA	TCTACCATGCTCTGCCTCGT
PtBMY4	POPTR_0003s08360	580143	eugene3.01180078	50.4% BAM4 (AT5G55700)	TCACCATACCTTTGGCATGA	GCTCACCCAAAAATGACGAG
PtBMY5	POPTR_0017s06840	679498	grail3.0064001202	71.4% BAM5 (AT4G15210)	ATTTGGTCTGCTTGGTGGTC	GCTCACTCATAGCCCTAGAAGG
PtSUC2.1	POPTR_0019s11560	249755	gw1.XIX.2155.1	77.9% SUC2 (AT1G22710)	CCCTCAAACAAAATGGCTGT	TGGCCTTTGATACATCACCA
PtSUT1.2	POPTR_0013s11950	287697	gw1.41.182.1	76.4% SUC2 (AT1G22710)	AGAGGGCTCCCACCAGTAGT	TCTGCTGCATCTCAAAAGGA
PtSUT2.a	POPTR_0008s14750	766309	fgenesh4_pg.C_LG_VIII001323	83.6% SUT2 (AT2G02860)	AACGGCCATGAACTTTCAAC	GCCATAACGACAAGCACTGA
PtSUT2.b	POPTR_0010s10360	769371	fgenesh4_pg.C_LG_X000861	79.1% SUT2 (AT2G02860)	AGCTCTCAAGCTGCCAAATC	TGGAACTCGGAAGAACCATC
PtSUT4	POPTR_0002s10710	830252	estExt_fgenesh4_pm.C_LG_II0488	72.4% SUT4 (AT1G09960)	GTATTGCGTCCGCTTACCAT	TTGGCGTAACGGGACTCTAC

Figure legends

Figure 1

Physiology of plants following artificial induction of embolism. (A) Extent of embolisms represented as percent loss of conductivity (PLC) induced by the ~1.0 MPa air injection into stems of *P. trichocarpa*. (B) Stem water potential measured using balancing pressure method on non-transpiring leaves. (C) Starch content in wood expressed per tissue fresh weight. (D) Sucrose content in wood expressed per tissue fresh weight. In each case (A-D) control plants are represented by the black column and air injected plants by the grey column. Control plants were treated the same way as plants with induced embolism including incision handling except application of air pressure. Each histogram is the mean of six plants and error bars represent SE. Stars denote significant difference with $p<0.05$ between treatment and control plants.

Figure 2

Relative expression profile of aquaporin (PIP1-PIP2), α-β amylases (AMY-BMY) and sucrose transporter (SUT/SUC) genes in the wood of *P. trichocarpa*. The transcript level of each gene in wood of treated plants was color coded as the relative expression (log fold changes) to the level of that gene expression of respective control plants. The results are the averages of three independent biological samples with three technical replicates. Letters indicate level statistical significance of reported change in expression where (a) indicates $p<0.1$, (b) $p<0.05$ and (c) $p<0.01$. A-section shows the results from air injection experiment (0.5 and 2 hours following the treatment), B-section shows the results from application of different osmotica (2 hours following the treatment), C and D-panels

shows the results from sucrose (SUC) and glucose and fructose (G+F) applications (0.5, 2 and 4 hours following the treatment) and recovery treatments (Rec) where control conditions were applied after two hours of treatment for an additional two hours.

Figure 3

Effect of application of monosaccharides (fructose and glucose), sucrose, PEG, and KCl on starch concentration (expressed per tissue fresh weight) in woody parts of *P. trichocarpa* stems. Plants with shaved root tips were exposed to a nutrient solution (control) or nutrient solution supplemented with the listed substances and the osmotic equivalent of ~1.0 MPa. Each histogram is the mean of six plants and the error bars represent SE. Stars denote a significant difference with $p<0.05$ between treatment and control plants (2 hour following the treatment).

Figure 4

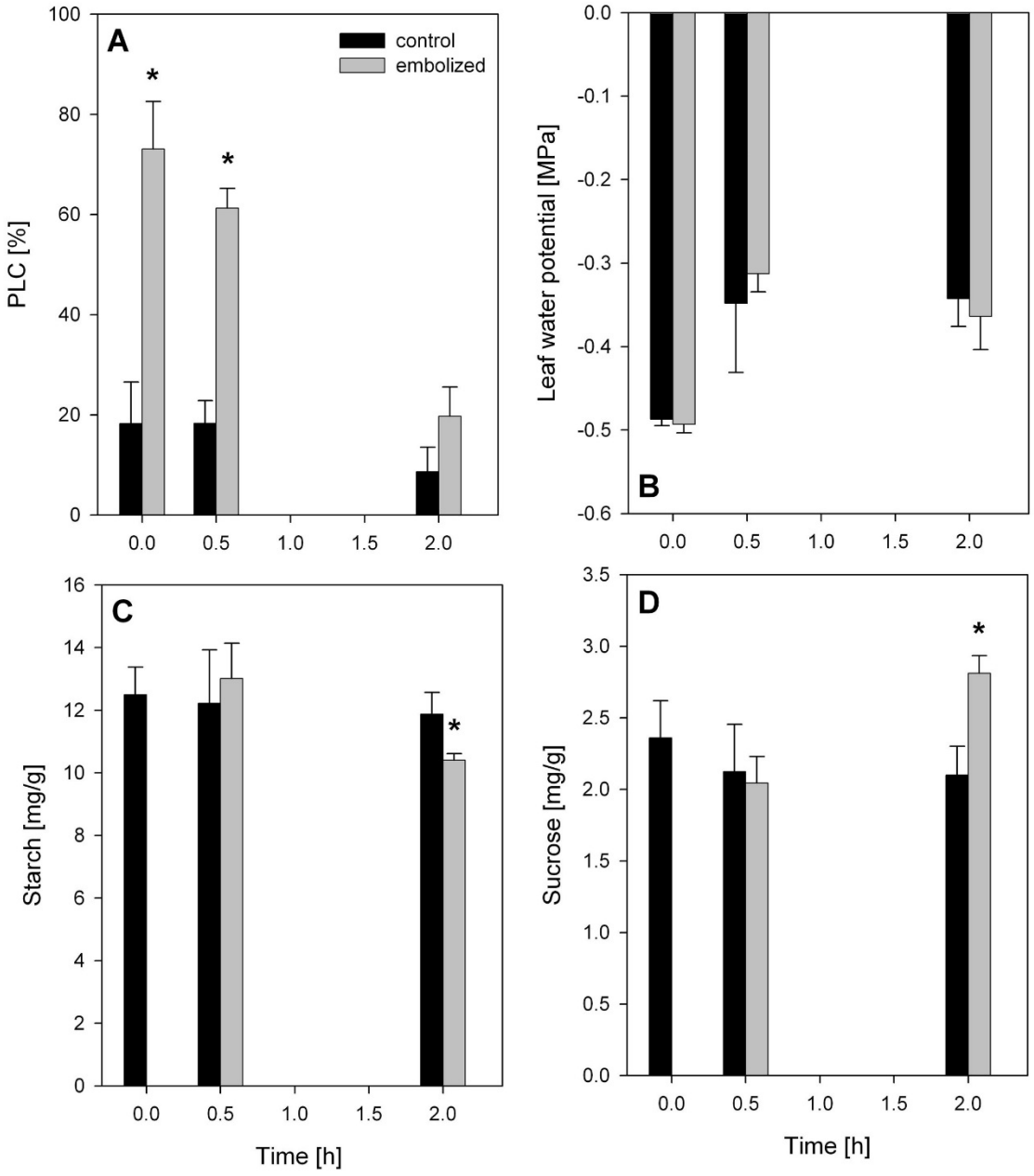
Temporal effect of application of monosaccharides (fructose and glucose) and sucrose on starch content in wood of *P. trichocarpa*. Plants with shaved root tips were exposed to nutrient solution (control) or nutrient solution supplemented with respective sugars in the osmotic equivalent of ~1.0 MPa. Wood samples were harvested after 0, 0.5, 2 and 4 hours exposure to the treatments. The data are the averages of three plants for each time and treatment and error bars represent SE. Stars denote significant differences with $p<0.05$ between treatment and control plants (for particular time period).

Figure 5

Comparative analysis of co-expression of all 20 genes (grouped by gene families PIP1 – 5 genes, PIP2 – 4 genes, AMY – 3 genes, BMY – 4 genes and SUC – 4 genes including

591 SUT) analyzed in this study between the air injection experiment (x-axis) and xylem
592 infiltration experiments (y-axis), two hours following the respective treatment. Values are
593 presented as relative expression (log fold changes). Regression lines are presented with
594 95% confidence intervals. Red lines denote presence of statistically significant
595 correlation ($p<0.05$).
596
597 **Figure 6**
598 Schematic representation of embolism detection by sucrose. Accompanying events are
599 explained on the left side of the three (A-C) experimental scenarios. White arrows
600 indicate flow of sucrose in walls toward the xylem. Dotted lines indicate sensing of the
601 sucrose near the cellular membrane and transduction of the signal to parenchyma cells.
602

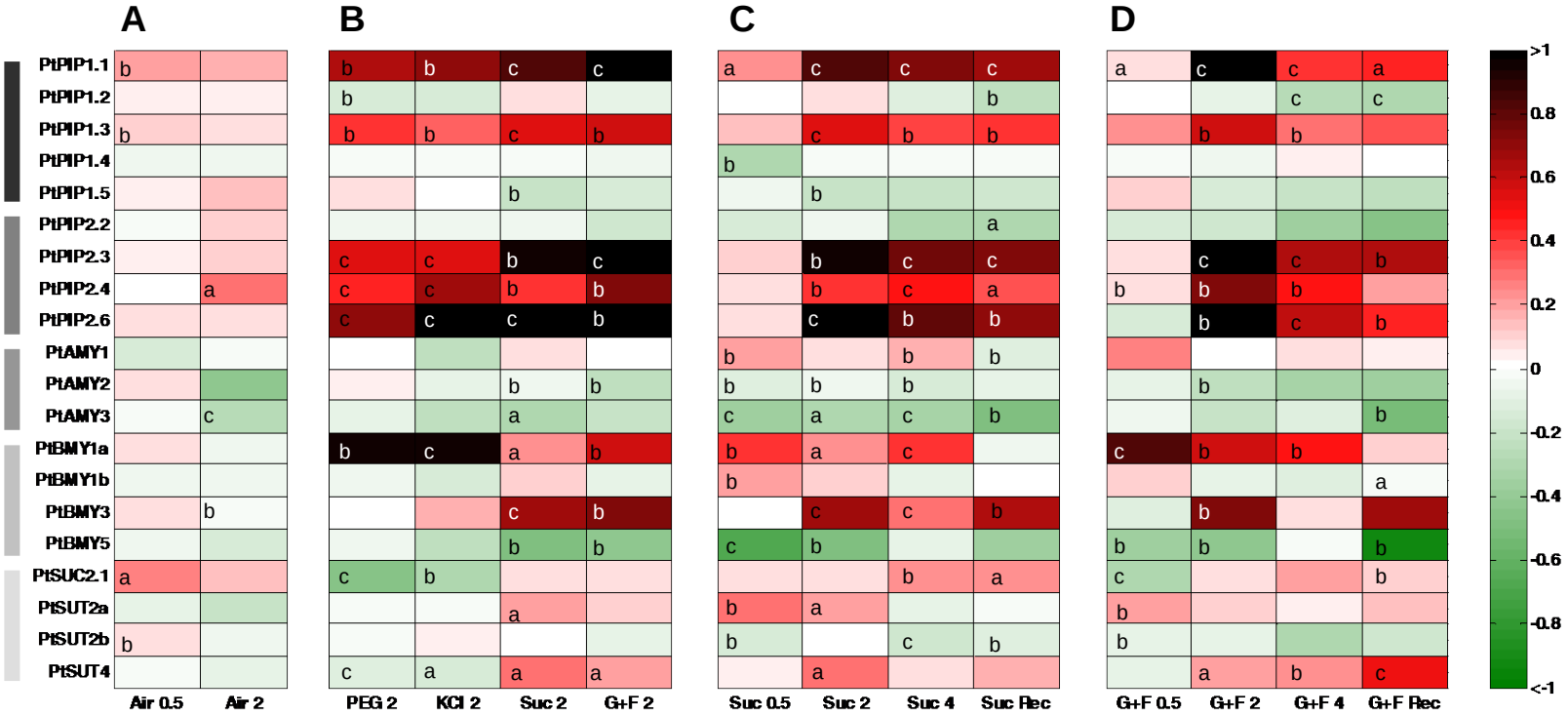
603 Figure 1



604

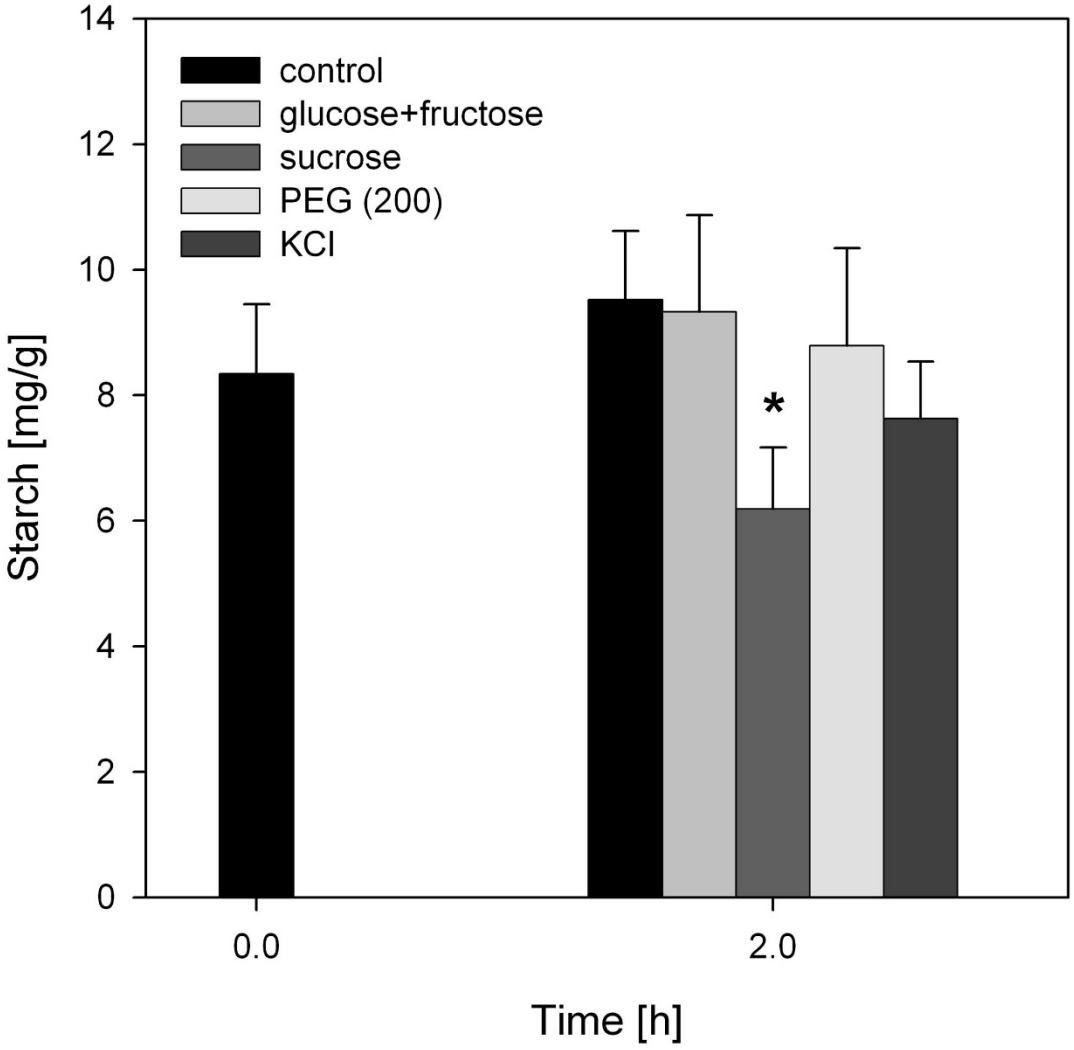
605

606 Figure 2



607

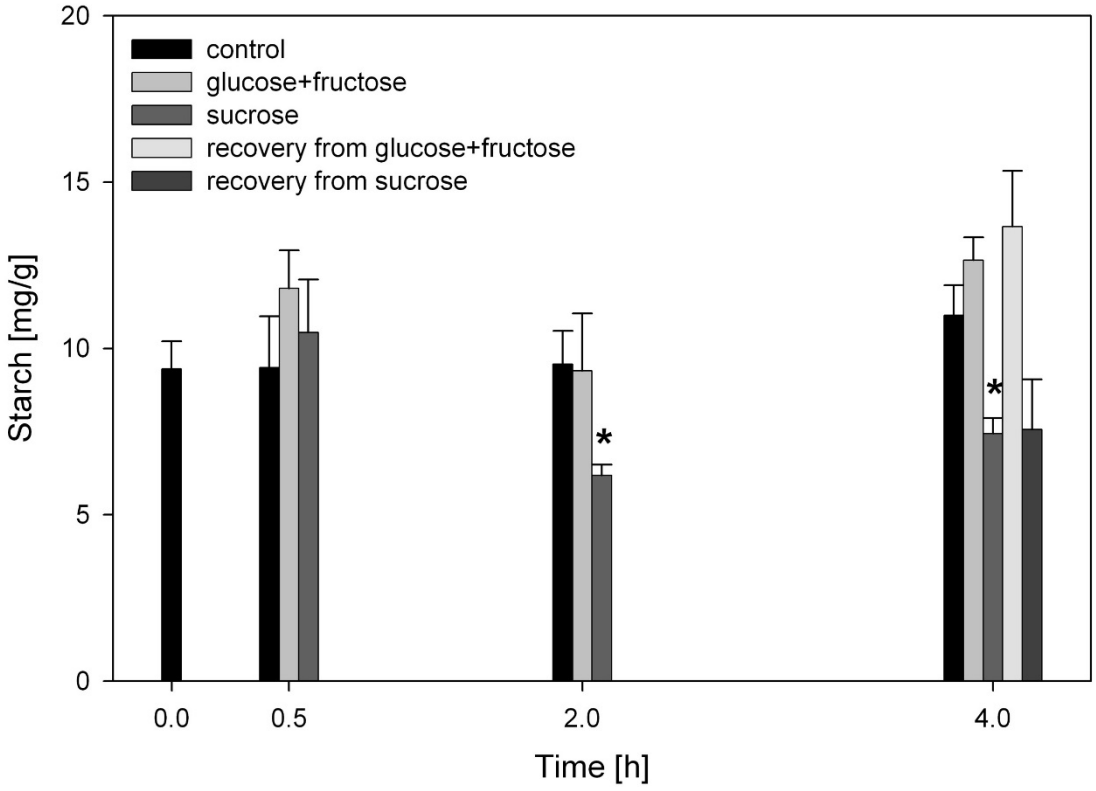
608 Figure 3



609

610

611 Figure 4



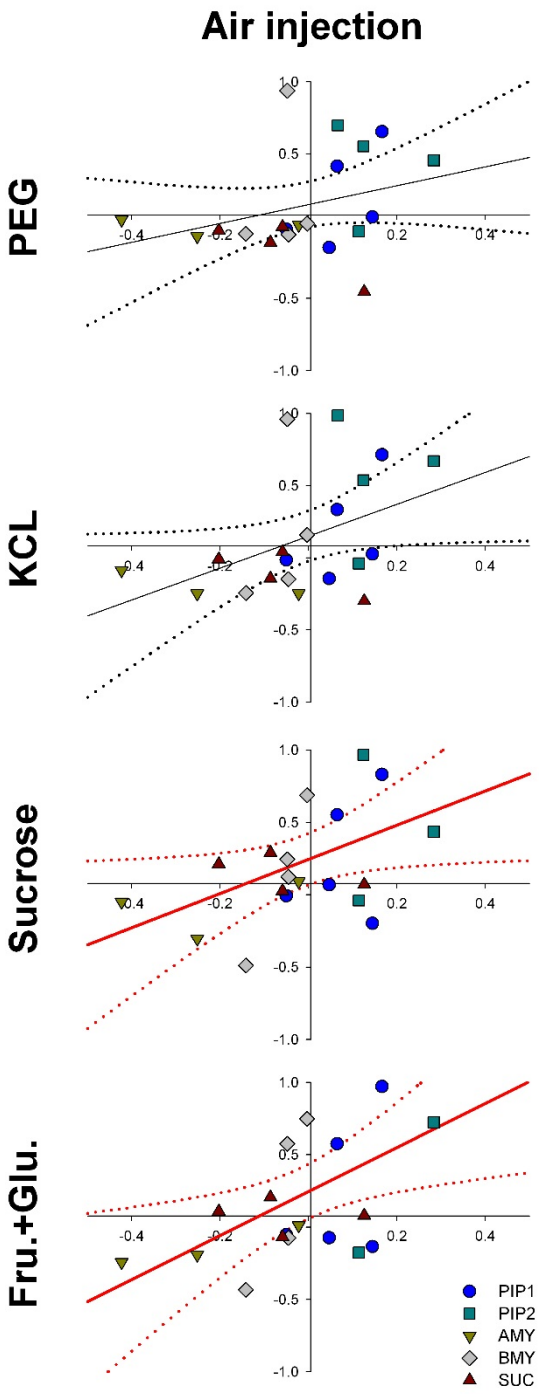
612

613

614

615 Figure 5

616



617

618 Figure 6

619

A. Functional vessel:

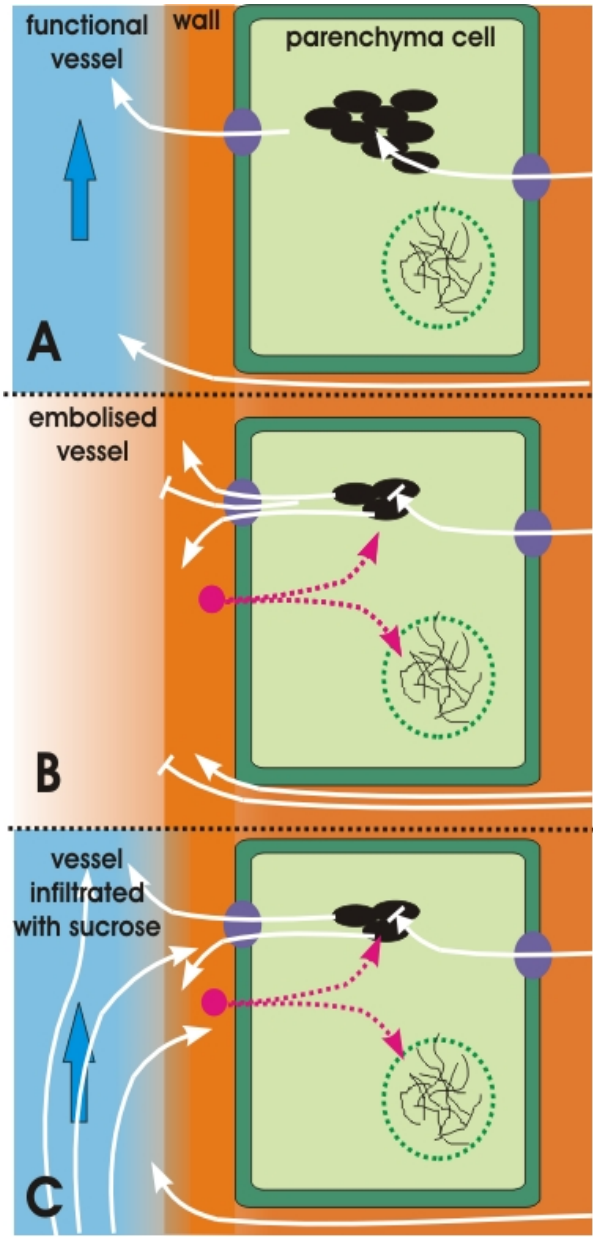
- Sucrose leaks from phloem and/or parenchyma cells (white arrows)
- It is scavenged by the parenchyma cells and used to form starch
- Its excess is carried away with the transpirational stream
- There is low concentration of sucrose in cellular walls
- Parenchyma cells are in ‘passive’ mode

B. Embolized vessel:

- Sucrose leaks from phloem and/or parenchyma cells (white arrows)
- It rapidly accumulates in the walls
- High sucrose concentration triggers ‘repair’ mode in parenchyma cells (starch degradation and specific expression pattern of genes – dotted arrows)
- Return to functional state ‘A’ allows for removal of sucrose excess and reduction of its concentration. Low sucrose concentration sends ‘off’ signal and return of cells to ‘passive’ mode

C. Artificial treatment of stem with sucrose:

- Sucrose leaks from phloem and/or parenchyma cells (white arrows)
- High sucrose concentration in sap exceeds the threshold for triggering the ‘repair’ mode.
- Degradation of starch in xylem parenchyma cells and pattern of gene expression is similar to that caused by embolism (dotted arrows)



620