Accumulation of sugars in the xylem apoplast observed under water stress conditions is controlled by xylem pH

Accumulation of sugars in the xylem apoplast observed under water stress conditions is controlled by xylem pH.

Short running title: Xylem pH regulates sugar accumulation in apoplast

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Abstract

Severe water stress constrains, or even stops, water transport in the xylem due to embolism formation. Previously, the xylem of poplar trees was shown to respond to embolism formation by accumulating carbohydrates in the xylem apoplast and dropping xylem sap pH. We hypothesize that these two processes may be functionally linked as lower pH activates acidic invertases degrading sucrose and inducing accumulation of monosaccharides in xylem apoplast. Using a novel *in vivo* method to measure xylem apoplast pH, we show that pH drops from ~6.2 to ~5.6 in stems of severely stressed plants and rises following recovery of stem water status. We also show that in a lower pH environment, sugars are continuously accumulating in the xylem apoplast. Apoplastic carbohydrate accumulation was reduced significantly in the presence of a proton pump blocker (orthovanadate). These observations suggest that a balance in sugar concentrations exists between the xylem apoplast and symplast that can be controlled by xylem pH and sugar concentration. We conclude that lower pH is related to loss of xylem transport function, eventually resulting in accumulation of sugars that primes stems for recovery from embolism when water stress is relieved.

Keyword index: in vivo pH, recovery, sugar accumulation, xylem apoplast

Introduction

Responding to continuous changes in soil water availability and atmospheric evaporative demand is a major part of plants' daily physiological activity. To ensure species survival in a given environment, a plant's transport system is expected to provide relatively uninterrupted supply of water for transpiration (Holbrook & Zwieniecki, 2008, Sperry, 2003, Sperry *et al.*, 2003). However, long-lived trees may be exposed not only to these daily changes but also randomly occurring periods of severe drought that result in loss of xylem function (Holtta *et al.*, 2009, Secchi & Zwieniecki, 2010, Tyree & Sperry, 1989, Zwieniecki & Holbrook, 2009). While recovery of xylem function may not be feasible under drought, increased water availability (rain, fog, snow etc.) followed by reduction in xylem tension may provide necessary stress relief to allow for restoration of xylem hydraulic capacity to pre-stress levels (Burgess & Dawson, 2004, Laur & Hacke, 2014, Mayr *et al.*, 2014).

Successful hydraulic recovery might depend on plant physiological activity during the onset of stress that 'primes' the stem for fast recovery under short-term temporary relief from severe stress. 'Hydraulic priming' might include biological activity of stem parenchyma cells that respond to loss of xylem transport function (i.e. cessation of transport) or direct presence of embolism (Brodersen & McElrone, 2013, Chitarra *et al.*, 2014, Nardini *et al.*, 2011, Salleo *et al.*, 2004, Salleo *et al.*, 2009, Zwieniecki *et al.*, 2004). Recent studies describe changes in expression levels of genes in parenchyma cells responding to artificially induced embolism. Observed changes included up-regulation of carbohydrate metabolism enzymes (including the starch degradation pathway), sugar transporters, ion transporters and aquaporins (Secchi *et al.*, 2011, Secchi & Zwieniecki, 2010). Such a systemic response suggests physiological significance of the biological processes triggered by loss of xylem function.

Analysis of *Populus nigra* xylem sap chemistry associated with onset of water stress revealed specific changes in parenchyma cell physiological activity that dropped apoplastic pH and increased sugar concentration in the xylem sap (Secchi & Zwieniecki, 2012). Significant differences in pH were especially noticeable between sap collected from functional and non-functional vessels (the latter is presumed to be isolated from transpiration stream by embolism at distal location), showing values of pH ~6.5 and ~5.5 respectively. In addition to observed pH differences, sugar concentration in xylem sap collected from non-functional vessels was also significantly higher than in vessels that remained functional [(Secchi & Zwieniecki, 2012) and Fig. S1]. Although sugar concentrations could not generate pressure sufficient to counteract hydrostatic tension in the xylem and allow for xylem refilling under water stress ($\Psi < -0.2$ MPa), they were within the range required for restoration of xylem function upon relief from water stress following re-watering of the trees ($\Psi > -0.2$ MPa) (Secchi & Zwieniecki, 2012). Taken together, observed xylem sap chemistry and parenchyma cell gene expression support the hypothesis that increasing stress and loss of xylem transport result in parenchyma cell activities that prime stems for subsequent, fast recovery of xylem function upon plant rehydration to pre-stress levels.

Our recently proposed model provides a hypothetical explanation for the observed changes in xylem sap chemistry (please consult Secchi & Zwieniecki, 2012). Briefly, we first assume that water stress (i.e. embolism) increases the starch degradation rate (Salleo *et al.*, 2009) resulting in higher cellular sucrose concentration, a notion supported by the increased expression of enzymes from di-carbohydrate metabolic pathways that include starch degradation enzymes (Secchi *et al.*, 2011). Increasing symplast sucrose concentration may shift the membrane sucrose gradient allowing for the sucrose efflux into apoplastic compartments via sucrose H-coupled transporters

as was recently shown experimentally (Geiger, 2011, Wippel *et al.*, 2010). Although most of the sucrose transporters have been characterized as H^+-sugar importers, recently electrophysiological studies of sucrose-induced current of ZmSUT1 in oocytes revealed an alternative transport mode showing that this transporter can also mediate the active efflux of sucrose. The authors showed that ZmSUT1 protein catalyzed H+-coupled sucrose symport and that the transport depended on the direction of the sucrose and pH gradient as well as the membrane potential (Carpaneto *et al.*, 2005, Carpaneto *et al.*, 2010). It is worthwhile to note, however, that the biochemical properties of plant sugar transporters have been studied in heterologous systems rather than in plants. I*n planta*, transgenic potato plants with downregulated expression of StSUT1 showed a lower tuber yield when phloem unloading towards tubers was apoplastic, indicating a major role for StSUT1 in efflux towards sink organs (Kuhn *et al.*, 2003). However, the proof of a dual mode of H^+-sucrose transporters in living xylem parenchyma cells has to be clearly demonstrated yet.

As sucrose efflux is accompanied by proton efflux, apoplastic pH is expected to drop concurrent to rising sucrose concentration. A drop in pH from ~6.2 [a typical xylem sap operational value based on the average of 22 perennial species (Sharp & Davies, 2009) and poplar (Secchi & Zwieniecki, 2014)] to below 5.8 would result in an increase of apoplastic invertase (i.e. acidic invertase) activity. Invertase is responsible for hydrolyzing sucrose into glucose and fructose, thus promoting the reduction of the disaccharide concentration and at the same time increasing the production of the monosaccharides. This process would result in lower apoplast sucrose concentration, thus maintain the symplast-apoplast sucrose gradient that consequently would lead to a continuous sucrose efflux. However, a continuous drop of apoplastic pH would eventually slow the efflux as sucrose co-transporters are highly sensitive to the pH gradient (Bush, 1990, Carpaneto *et al.*, 2005, Geiger, 2011, Wippel *et al.*, 2010). Eventually, a new

106 homeostasis of pH and sucrose gradient between cells and apoplast would be achieved, where
107 the sucrose gradient is maintained by apoplastic invertase activity and by the symplastic starch
108 degradation rate. Further, the new pH gradient would be constrained by both proton efflux via
109 sucrose transporters and influx of protons to the symplast via ion anti-porters and membrane
110 proton pumps.

111 Here, we aim to directly test part of the above hypothesis-scenario that relates xylem
112 apoplast pH to sugar accumulation. First, we provide direct evidence of xylem apoplast pH
113 changes in response to water stress using a novel experimental setup that allows for *in vivo*
114 determination of pH in tree stems. Further, we provide evidence from *in vitro* analysis of
115 changes in sugar concentration in xylem sap in response to different apoplastic pH levels.

116

117 **Material and Methods**

118 *Plant materials*

119 Experiments were conducted on 1-year-old plants of hybrid white poplars (*Populus tremula* x
120 *Populus alba*; Institut National de la Recherche Agronomique France clone 717-1B4). In total,
121 28 poplars were used in two different experiments. Poplar were grown either two plants per 1L
122 pot (*in vivo* experiment) or one plant in 4L pots (*in vitro* experiment) filled with potting mix.
123 Plants were grown in a glasshouse with the following ambient conditions: temperature
124 maintained in the range of 25–32°C and natural daylight supplemented with light from metal
125 halogen lamps to maintain a minimum of 500–600 µmol photons m^{-2} s^{-1} during a 12-h-light/12-
126 h-dark cycle. Plants were approximately 1.2-1.5 m tall at the onset of the two experiments:

127 (1) *In vivo* experiment aimed at analysis of pH changes in response to increasing water stress and
128 recovery. Six plants were used in this study. Two plants were grown in one pot (1L size) under

no stress conditions (watered to field capacity every day) and then subjected to drought by withholding irrigation. Small pots allowed for induction of severe drought within 2-3 days. The duration of severe drought was limited to 24 hours and plants were then re-watered. During the entire time from stopping irrigation to final recovery, one plant in a pot (from two-plant system) was used to determine stem water potential (see methods below) while a different plant from the same pot was used to determine xylem apoplastic pH (see methods below). This procedure was utilized to avoid wounding of the plant that pH was measured on. Through the entire treatment from withholding irrigation, plants were kept in low-level light (~200 μmol m^{-2} s^{-1} of PAR).

(2) *In vitro* experiment aimed to determine the kinetics of sugar efflux in response to different pH buffers. Twenty-two poplars were used in this study. Plants were never exposed to severe water stress.

Measurements of stem water potential

Stem water potential was measured on non-transpiring leaves. Leaves were covered with aluminum foil and placed in a humidified plastic bag for at least 15 minutes prior to excision. After excision, leaves were allowed to equilibrate for an additional 20 minutes before the xylem pressure was measured using a Scholander-type pressure chamber (Soil Moisture Equipment Corp., Santa Barbara, CA, USA).

In vivo determination of xylem apoplast pH

Xylem apoplast pH was determined using pH sensitive dye (carboxyfluorescein diacetate - CFDA) conjugated to 15 kD dextran. The 15 kD size dextran was used to provide assurance that dye would not be taken up by the living cells as the goal of the study was to determine xylem

apoplastic pH. CFDA was conjugated to dextran in the lab following the procedure described by de Belder and Granath (1973). CFDA dye provides a good estimate of pH for the range of 4 to 7 determined as a ratio of fluorescence measured at 510 nm excited by 495 and 430 nm respectively (Geilfus & Muehling, 2011).

An 'in-lab' developed tool was built to determine fluorescence ratio (Fig. 1A). The system consisted of a sensor head attached to the plant with two fiber optics guides attached to it. One of the fiber optics was bifurcated with one end attached to a 430 nm light emitting diode (LED) and second end to a 495 nm LED, both narrow band (+/- 5 nm). A second fiber optic cable collected reflected and fluorescent light and was linked to high gain photodiode via a narrow band 510 nm (+/- 2 nm) filter (Fig. 1A). LEDs were controlled by a computer and turned on consecutively such that only one LED illuminated the stem at a time. Fluorescence was measured by a lock-in amplifier (International Light LI-1700) and data collected by a dedicated computer. To avoid photo bleaching of the dye in long-term experiments, we used low-level irradiance and controlled exposure time to few seconds measured once per hour.

Initial validation of system performance was done on micro-capillary tubes (5 uL) filled with range of pH buffers (4.93 to 7.03 pH). Buffers were mixed with CFDA-dextran conjugate to a final concentration of 4 mg per mL of dye and fluorescence was measured (Fig. 1B.1). This test was followed by testing dye-dextran conjugate response to pH directly applied to poplar stems (Fig. 1B.2). Stems were prepared by removing a small section of bark with phloem (3x6 mm) and exposing xylem (Fig 1A.1-3). The end of each stem section was connected via silicone tube to a manifold allowing for perfusion of a series of pH buffers (4.93-7.03) (Fig. 1A.5). Two techniques were tested (1) dye-dextran conjugate was applied directly to the xylem and (2) dye-dextran conjugate was soaked onto a small piece of filter paper (size of exposed xylem) that was

hydraulically connected to the xylem via 1.5% agarose gel (Fig. 1A.1-2). In both cases, the window in the bark was covered with a transparent plastic sheet sealed to the stem via silicone vacuum grease to remove the possibility of xylem surface drying (Fig. 1A.3). Final concentration of dye used in stem tests and all subsequent analysis was 20 mg of CFDA/Dextran conjugate per one gram of water. Large size (15kD) of the dye-dextran conjugates limited diffusion of the dye into the stem or from the filter paper to the stem while allowing for stem sap to diffuse freely.

Following the preparation procedure, pH buffers were consecutively perfused through the stems using low pressure (15kPa) and the fluorescence ratio measured on either soaked xylem or dye-soaked filter paper (Fig. 1B). The pH of the buffer perfused through the stem was determined and used in the calibration. In all cases a relatively good correlation between pH and a fluorescence ratio was observed, although ratios were specific to each stem (problem most likely related to uneven dye application or surface properties of observed field). This specificity resulted in the need of fluorescence/pH calibration for each stem following each *in vivo* measurement (Fig. 1B). In general, the filter paper approach produced a stronger fluorescence signal and a less variable calibration curve, although it also resulted in several minute delays in response time after switching of pH buffer when compared to the direct application of dye to the xylem. This delay was probably due to time required for buffer diffusion from xylem to filter paper through the agarose gel. As this delay was relatively short compared to the expected duration of experiment (minutes vs. hours), the 'filter paper' approach was selected for long-term experiments due to its stronger signal. Once placed on the stem, filter paper was not removed until conclusion of the experiment. Although signal strength was observed to diminish over time, it remained measurable up to 5 days (when test was stopped, it is possible that setup could be

used for even longer time). It is important to note that xylem window has to be perfectly sealed for gel junction to remain wet and thus provide the hydraulic connection between xylem and filter paper through entire experiment duration.

The calibration curve from the micro-capillary tubes could be fitted with a linear model (df=6, R^2=0.976, SS=13.73) or a four-parameter sigmoid curve for the range from ~4.93 to ~7.03 pH (df=4, R^2=0.9991, SS=13.74), (Fig. 1B.1). An F-test (F=0.002) did not suggest that the sigmoidal model provided a significantly better fit for a given pH range. Similarly, stem calibration curve could be fitted with linear model (df=6, R^2=0.993, SS=13.74) or four parameter sigmoidal model (df=4, R^2=0.996, SS=13.75), (Fig, 1B.3); again F-test (F=0.002) did not suggest that sigmoidal model provided a significantly better fit for a given pH range. Thus, the linear model was used for the calibration line using two buffers (4.93 and 7.03) that were perfused through the stems of experimental plant at the conclusion of the stress experiment for 60 minutes each (allowing for equilibration time). Calibration was performed without touching the measuring setup. The stem was cut above and below the fluorescence measuring point and the upper end of the stem was connected via a silicone tube to a manifold (as done in the stem tests – see above).

In vitro experiments to determine kinetics of sugar efflux in response to apoplastic pH

A total of 22 plants were selected. From each plant, six segments of branches with similar length and diameter were cut (length: 76.75 mm ± 7.82 and diameter: 5.10 mm ± 0.77). We removed 0.8 cm of bark from both sides of the segment and wrapped entire segments with Parafilm sheets to avoid evaporation. An ‘in-lab’ sap perfusion tool was built to run the experiment (see Fig. 1C). Both ends of the branch segments were fixed to silicone rubber tubing with volume of ~1mL (Fig. 1C.2). On one end tubes were closed, while on the other end tubes were connected to

a channel that allowed air pressure application (Fig. 1C.3). Tubes were filled with different buffer solutions or DI water (volume of 1 mL per stem). Once filled with the liquid, tubes were pressurized with 0.15 MPa for 15 sec every 90 sec to allow buffers to perfuse throughout the segments to the closed side and upon pressure release to return to the initial position. The perfusion system was submerged in water to prevent stem desiccation. At the pressurization site of the system each tube had an access channel allowing for the collection of the liquid sample from water being perfused through the stem segment. During the experiment 40 µL of solution was collected from a single channel linked to individual segment after 10, 30, 60, 120 and 180 minutes. The liquids collected were immediately stored at -80 °C.

To assess how pH influences the kinetics of sugar accumulation (exchange between xylem parenchyma and xylem sap), stem segments were subjected to four different treatments:

1- pH 4.5 buffer (100 ml of 0.1 M Potassium hydrogen phthalate, 17.4 ml of 0.1 M Sodium hydroxide, 82.6 ml of distilled water)
2- pH 6.5 buffer (100 ml of 0.1 M Potassium dihydrogen phosphate, 27.8 ml of 0.1 M Sodium hydroxide, 72.2 ml of distilled water)
3- DI water
4- DI water plus sodium orthovanadate, (BioLabs, New England, MA) used at concentration of 1.0 mM. Sodium orthovanadate is an inhibitor of many membrane P-type pumps, among which the plasmalemma proton pump.

Soluble carbohydrate content and pH measurements in the efflux liquid in response to apoplastic pH

The anthrone-sulfuric acid assay (Leyva *et al.*, 2008) was used to quantify soluble carbohydrate content in the liquids. The anthrone reagent was prepared immediately before analysis by dissolving 0.1 g of anthrone (0.1%) in 100 mL of concentrated sulfuric acid (98%).

Briefly, 150 µl of anthrone reagent was added to each well of the microplate containing 50 µL of standard solutions (see below), sample solutions and water. Plates were kept for 10 min at 4 °C. Then, plates were incubated 20 min at 100 °C. After heating, plates were cooled for 20 min at room temperature and absorbance at 620 nm was read with a microplate reader (Multiscan Thermo Scientific). Colorimetric response was compared to the glucose standard curve (0, 0.01, 0.03, 0.1, and 0.3 g L^{-1} glucose) and total carbohydrate content was calculated as mg/mL of glucose. Analysis of sugars using the anthrone method provided results similar to those obtained through the high-performance liquid chromatography (HPLC) technique, as shown in Fig. S2, and confirming the reliability of both methods. The pH of each sample was measured using a micro pH electrode (Thermo Scientific, Beverly, MA, USA).

Analyses of sucrose, glucose and fructose

After thawing, the liquid samples were centrifuged at 15,000 × g for 10 min. The supernatant was filtered through a 0.2 µm syringe filter (Millex-HP filter, Millipore Co., MA, USA) and collected in 1 ml glass vial and used for high-performance liquid chromatography (HPLC) analysis. Reverse phase-liquid chromatography (Agilent HPLC 1100 series, USA) was performed to determine individual sugar content (sucrose, glucose and fructose). Separation of sugars was performed with water as a mobile phase flowing at 0.6 ml min^{-1} using an Aminex HPX-87C column (300 mm × 7.8 mm; Bio Rad Laboratories, Hercules, CA, USA), which was preceded by a micro-guard cartridge (Carbo-C, pH range 5–9, 30 mm × 4.6 mm; Bio Rad

Laboratories, Hercules, CA, USA) and maintained at 80 ºC. A ten-microliter extract was injected by an auto-sampler and sugars were detected using a refractive index detector (Agilent G1362A). Chromatographic peaks were identified by comparing retention times with those of standards and by spiking samples with pure compounds while quantification was carried out using the external standard method. The concentrations of different sugars were expressed as mg/ml of fluid.

Results

In vivo determination of xylem pH using pH sensitive dye (carboxyfluorescein diacetate - CFDA) fused to 15kD dextran suggests that *Populus tremula* x *Populus alba* xylem operated at relatively constant value of pH (~ 6.2) across wide range of water stress levels. In poplars experiencing a severe drought (stem water potential below -2.5 MPa) a significant drop in xylem pH was observed (to the level of ~ 5.6), (Fig. 2B). The drop in pH coincided with turgor loss in leaves (Fig. 2A) and would coincide with significant loss of stem hydraulic conductivity with the percent loss of conductivity exceeding 80% for this level of stress (Secchi & Zwieniecki, 2014). Re-watering of the plants resulted in almost immediate (less than 2 hours) restoration of leaf turgor and in recovery of stem water potential to pre-stress values within 12 hours. Surprisingly, xylem pH lagged recovery of water potential for almost 24 hours before the pre-stress level of ~6.2 pH was reached (Fig. 2).

In vitro exposure of xylem to two levels of pH using buffers (4.5, 6.5) and to DI water resulted in significant changes of soluble sugar concentration in the liquid (Fig. 3). In all three treatments a fast increase in sugar content was observed over initial period of 10-20 minutes as both buffers and DI water applied were free of sugars (Fig. 3A). In the case of pH 6.5 buffer the sugar concentration plateaued very fast at the level of *c.* 0.2 mg/ml. Such plateauing was not

observed in the low pH 4.5 treatment over 3 hours of experiment duration; instead a continuous increase of sugar concentration was present reaching a content of 0.5 mg/ml at the end of the experiment (Fig. 3A). Acidity of the pH 6.5 buffer treatment stayed relatively constant over the duration of the experiment (~pH 6.2), while pH of the 4.5 buffer slowly rose, eventually reaching value of 5.15 pH (Fig. 3B). Estimate of the maximum pH from fitting available time trend data with the exponential rise to maximum function was ~5.6 pH (fitting statistics - R^2=0.95, $p<0.001$); a value close to that observed in the *in vivo* pH determination (Fig. 2B). DI water treatment had relatively steady pH within the range of 7-7.5 (Fig. 3B) with sugar accumulation similar to that of ~6.5 pH buffer. The concentration of the sugars measured in the xylem at the end of each treatment was related to final pH values of the solution and the highest carbohydrate content was found at pH ~5 and low concentrations across the entire pH range from ~6.2 to 8.2 (Fig. 4A). Rate of sugar accumulation during last hour of the *in vitro* experiment was near zero in the range of pH 6.2 to 8.2 and it was still strongly positive in pH *c.* 5-5.2 (Fig. 4B).

Analysis of the basic sugar composition (sucrose, glucose and fructose) in the perfused liquid revealed that in 4.5 pH treatment the sucrose concentration was very low (almost absent at the end of the treatment), while concentration of fructose and glucose was high and continuously increasing during the duration of the experiment (Fig. 5A-B). By the third hour of the experiment fructose and glucose were dominant sugars with similar concentrations (Fig.5A-B). In both 6.5 pH buffer and DI water treatments sucrose was continuously present and its concentration was not rising in the third hour (there was no significant difference in sucrose content over the duration of the experiment), (Fig. 5C-E). Concentrations of glucose and fructose were also stable over this time period suggesting that the system reached equilibrium and no net carbohydrate efflux was present from parenchyma cells (Fig. 5D-F).

Sodium orthovanadate ($NaVO_3$) treatment was applied to determine if plasma membrane proton pumps were directly involved in the recirculation of protons necessary to keep the pH gradient and to energize the sugar transporters. Unfortunately, the test only could be done in DI water as vanadate salt interacted with applied buffers and was previously shown to be unreliable in acidic environments (Vreugdenhil & Spanswick, 1987). In DI water, vanadate presence significantly reduced the apoplastic sugar accumulation. The efflux level was inhibited by 27% after only 10 minutes and maintained similar level of inhibition until the end of third hour with values ranging from 36 to 41% (Fig. 6A). The pH of the DI water and pH of $NaVO_3$ solution changed in a similar manner over the duration of the experiment and was never different between two treatments at the same time (Fig. 6C). Thus, the observed reduction in sugar concentration can be linked directly to the presence of vanadate and its effect on proton availability for sucrose transporter activity (Fig. 6B).

Discussion

In vivo determination of xylem apoplast pH in *P. tremula x alba* in response to the onset of water stress reveals its relative homeostasis around a pH value of 6.2. This level was maintained until stem water potential reached -2.5 MPa. Continuous stress that resulted in leaf turgor loss coincided with a fast (~7 hours) drop of pH to a new level of ~5.6, which was later maintained despite a slower drop in stem water potential. As stress below -2.5 MPa was also shown to be correlated with >80% loss of conductivity in this species (Secchi & Zwieniecki, 2014), we suspect that the observed changes in pH might reflect a shift from mostly functional transpiring xylem (with active transpirational water stream) to mostly non-functional xylem (either embolized or hydraulically isolated from flow by embolism presence in distal locations, or xylem

characterized by loss of transpirational activity which would reflect leaf turgor loss and transpirational cessation due to stomatal closure). Plant rehydration did result in fast recovery of leaf turgor (2 hours) and stem water potential (12 hour) to pre-stress condition. However, xylem pH did not start to recover until 24 hours after the return of plant water potential to pre-stress conditions (36 hours after re-watering). The length of delay in recovery of pH is similar to the time necessary for the recovery of stem hydraulic function from severe stress as observed previously in this species [~24 hours; (Secchi & Zwieniecki, 2014)], further supporting the notion that low pH in non-functional stem is linked to loss of transport function (Secchi & Zwieniecki, 2012).

Both sucrose concentration gradient and pH differences were previously shown to be associated with sucrose transporter activity, ultimately affecting both the direction and rate of sucrose transport (Carpaneto *et al.*, 2005, Wippel *et al.*, 2010). Recently, a drop in xylem pH observed in non-functional vessels of poplar xylem (Secchi & Zwieniecki, 2012) was hypothesized to result from sucrose efflux occurring via sucrose/proton co-transporters. The lower pH has a positive effect on apoplastic invertase activity (Goetz & Roitsch, 1999) that effectively lowers apoplastic sucrose concentration, generating a sucrose concentration gradient that promotes sucrose efflux and accumulation of carbohydrates in xylem apoplast. The low apoplast pH effect on sugar accumulation in xylem apoplast is supported by our results where pH of ~4.5 led to continuous sugar accumulation in the xylem sap, mostly in the form of glucose and fructose (Fig. 3 and 5). We can further deduce that the efflux is mostly in the form of sucrose as higher pH (water and buffer) resulted in lower overall sugar concentration, but limited accumulation occurred in the form of sucrose (i.e. there was presumably very limited apoplastic invertase activity (Goetz & Roitsch, 1999). In addition, overall efflux was halted within 1 hour

in high pH, suggesting that the sucrose concentration gradient was a major driver of initial efflux activity (Fig. 3).

As efflux of sucrose from cells is coupled with co-transport of protons, a decrease in pH as observed in our experiments should be expected. However, this was not the case in both high and low pH treatments. In the high pH treatment, no significant changes in pH were observed over the duration of the experiment, while in the low pH treatment a significant increase of pH occurred (Fig. 3). Such behavior suggests that sucrose-coupled proton efflux is counter-balanced by increased proton pump activity. As the introduction of sodium orthovanadate (known proton pump blocker) significantly reduced sucrose efflux while not changing pH of the apoplast, we suspect that proton pumps not only work to maintain pH homeostasis (or return to pre-treatment state) but also might be involved in sucrose efflux control, a hypothesis also suggested by (Vreugdenhil & Spanswick, 1987) where vanadate inhibits the recycling of protons that were co-transported with sucrose. Such proton pump activity might be important for control of carbohydrate management under stress conditions. Results from the orthovanadate application have to be to considered with caution as orthovanadate is a phosphate transition analog and therefore it could bind with (and block) any enzymatic process that depends on ATP hydrolysis. Thus, vanadate can probably be assumed to select for the H-ATPase only over periods of few minutes while during longer experiments it could affect all processes that depend on continuous turnover of ATP. However, even if only short time application is considered in our experiments (<10 minutes), the sugar efflux was already significantly reduced.

Taken together, our *in vivo* observations of pH changes under severe water stress conditions and the role of apoplastic pH in promoting sugar accumulation in the xylem support the hypothesis that apoplastic solute accumulation under stress conditions (Secchi & Zwieniecki,

2011) is ultimately controlled by xylem sap pH via acidic invertases and proton pump activity. While level of sugar accumulation may not allow for immediate recovery of xylem function under stress (Secchi & Zwieniecki, 2012), it could prime the xylem for accelerated restoration of xylem function upon return to hydrated conditions. We propose the following scenario of physiological events related to function of xylem parenchyma cells during onset of water stress and recovery (Fig. 7):

- Under normal hydration, xylem is functional and cells maintain near steady-state level of xylem sap pH. Such a situation continues even under moderate water stress, as suggested by our *in vivo* observation in conjunction with prior evidence that xylem sap pH changes in perennial plants experiencing stress which only partially closes stomata (Sharp & Davies, 2009). On average pH in such conditions is > 6, leading to very low invertases activity (Goetz & Roitsch, 1999), and sucrose flux direction is controlled by the low sucrose concentration in the cell due to higher starch accumulation, rather than pH (as high apoplastic pH promotes efflux).
- During severe water stress levels when embolism and/or stomatal closure occur, a plant would experience dramatic reduction in transpirational flow rates (Brodribb *et al.*, 2003, Buckley, 2005, Cochard *et al.*, 2002, McDowell *et al.*, 2008, Meinzer *et al.*, 2009, Nardini & Salleo, 2000, Urli *et al.*, 2013). This stop in transpirational flow would change the balance of carbohydrate fluxes in xylem, i.e. carbohydrates will not wash away in the transpiration stream, and the plant may experience temporary accumulation of sucrose in the apoplast that can trigger a cellular stress response promoting starch degradation (Secchi & Zwieniecki, 2011). Starch degradation results in increased cellular soluble sugar concentration (i.e. maltose and then sucrose), providing not only osmotic protection

404 from stress but also shifting the membrane sucrose gradient and triggering both passive
405 (via membrane) and possibly proton-coupled sucrose efflux (Carpaneto *et al.,* 2005,
406 Wippel *et al.,* 2010) as treatment with sodium orthovanadate (proton transport blocker)
407 seems to block this efflux. The proton coupled sucrose efflux might be responsible for
408 initial increase of apoplastic sucrose concentration and drop of pH. Drop in pH could
409 also be promoted by activity of H^+-ATPase proton pumps.

410 - Lower pH leads to a several-fold increase in apoplastic invertase activity that splits
411 sucrose to fructose and glucose and lowers sucrose concentration promoting its further
412 efflux either via sucrose co-transporters or passive efflux through the membrane.
413 Leveling of the apoplastic pH could be achieved by increased activity of ion anti-porters
414 that would not only stabilize new lower pH but also increase metal ion concentration as
415 previously seen (Secchi *et al.,* 2011, Secchi & Zwieniecki, 2012). This notion of pH
416 stabilization is supported by our *in vivo* measurements where pH stabilized *c.* 5.6, and
417 observed rise of pH in *in vitro* experiment (buffer 4.5 pH).
418 - Upon recovery from stress, sugars continuously accumulate in the xylem and pH remains
419 low until full xylem functional recovery occurs. This recovery of transport function and
420 the consequently removal of embolism is facilitated by both the high osmoticum level
421 present in the xylem and whole plant level reduction of plant water stress (Secchi &
422 Zwieniecki, 2012). As transpiration resumes, the cellular stress reaction is 'triggered off'
423 by washing away sugars and changing xylem pH as new water is delivered from roots.

424 In our proposed scenario, the recovery of stem functionality follows plant re-hydration,
425 facilitated by a 'priming' of the xylem to recovery during stress via accumulation of solutes in
426 the xylem apoplast.

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Figure legends

Figure 1

(A) Preparation of stem for *in vivo* pH measurements. (A1) A small section of bark and phloem is removed (~3x6 mm). (A2) Exposed xylem is immediately covered with filter paper of the same size as the exposed xylem. Filter paper is pre-soaked with CDFA-dextran conjugate (at a concentration of 20 mg/g of water) and 1.5% agarose gel. Thick layer of vacuum grease (Dow Corning) is smeared on the bark around the filter paper. (A3) A transparent plastic sheet is placed over the filter paper and sealed to stem via vacuum grease protecting filter paper from drying out. (A4) Prepared section of the stem is then covered with perfectly light-tight clip with both bifurcated fiber optic guide connected to two narrow band light emitting diodes (LED; wavelength 495 and 430 nm +/-5 nm) and separate optic guide linked to high sensitivity high gain photodiode (International Light) connected. Fiber optics ends are placed over the filter paper. LEDs are consecutively turned on for six seconds once every hour and the fluorescence measured using narrow band light filter (510 nm +/-2). Measurements were conducted over 100 hours without any alteration to the system. (A5) At the conclusion of the experiment, the stem was cut above and below the fiber optics attachment (without any disturbance to the setup) and a plastic tube was attached to upper part of the stem. Then two pH buffers (4.93 and 7.03) were perfused consecutively through the stem for 60 minutes each and fluorescence measurements performed to allow for stem-specific calibration. **(B**) Three calibration approaches are presented for the use of the pH sensitive dye (CFDA) conjugated to 15 kD dextran. (B1): Test of solution in capillary tube. Capillary tubes (5 μL) were filled with buffers of specific pH (4.93, 5.34, 5.66, 6.03, 6.21, 6.48, 6.79, and 7.03) mixed with CDFA-dextran conjugate (at a final concentration of 4 mg/g of water), sealed at both ends, and measured. (B2): Test on branch segment with exposed

xylem. In this experiment dye soaked directly to exposed xylem. (B3): Test on branch segment with exposed xylem. Dye soaked to filter paper fused to exposed xylem via 1.5% agarose gel on a 5 cm long stem segment. For approach B2) and B3) stem segments were consecutively perfused with pH buffers (4.93, 5.34, 5.66, 6.03, 6.21, 6.48, 6.79, and 7.03) prior to measurements. Dye on exposed xylem B(2) produced highly variable outcome, while filter paper approach B(3) resulted in strong signal to noise ratio and well-defined calibration curve for each stem (dotted lines represent sigmoidal four parameter fit and continuous lines represents two-parameter linear fit (see methods for statistical analysis)). **(C)** An 'in-lab' sap perfusion tool was built to determine the kinetics of sugar efflux in response to apoplastic pH. Around 7.5 cm long stem segments (C1) were connected via silicon rubber tubes (C2) to manifold (C3). Manifold on the right provided a cup to each stem while on the left it was consecutively pressurized and de-pressurized forcing liquid to flow back and forth through the stem. A glass capillary (C4) was used to collect sample of liquid being perfused through each stalk.

Figure 2

In vivo determination of xylem pH changes in response to water stress.

(A) Leaf turgor dynamics during increase in and recovery from water stress. **(B)** Xylem pH and stem water potential are plotted over the time. Blue arrow indicates the time of the addition of water (recovery). Black arrows represent the status of leaf turgor at the determined time.

Figure 3

Temporal changes of **(A)** nonstructural carbohydrate concentrations and **(B)** pH values in segments of stems incubated in pH 4.5- pH 6.5 buffers and in DI water for 3 hours.

One-way Anova tests suggest significant differences among the three buffer treatments and among the times of incubation for each treatment ($p<0.05$). Letters denote homogeneous groups based on the Fisher LSD method (lower-case letter, differences among treatments; upper-case letter, differences among times for the same treatment). Data are mean values and bars are SE.

Figure 4

(A) Final sugar concentration as a function of pH value measured at the end of the treatments. Inset: Average xylem sugar content measured at the end of each treatment as it relates to final pH values. **(B)** Final rate of nonstructural carbohydrate accumulation as a function of pH value measured at the end of the treatments. Inset: Average sugar accumulation rate as it relates to final pH. For both insets, one-way Anova tests suggest significant differences between treatments ($P<0.01$). Letters denote homogeneous groups based on Fisher LSD method. Data are mean values and bars are SE.

Figure 5

Sucrose, glucose and fructose content in portions of stems incubated for 3 hours in pH 4.5, pH 6.5 buffers and DI water. Data are mean values and bars are SE. Statistical analysis has been done for each sugar independently and the Anova analysis reveals the presence of significant differences ($p< 0.05$) among times. Letters denote homogeneous groups based on the Fisher LSD test.

Figure 6

Effect of sodium orthovanadate on nonstructural carbohydrate content expressed as **(A)** percent of inhibition; no differences are observed among different times of the treatment (Anova analysis,

p=0.311) instead the percent of vanadate inhibition is different to zero (t-test, ***, p=<0.001), and as **(B)** concentration. Presence of orthovanadate significantly reduced sugar accumulation in xylem sap in each time of the treatment when compared with samples treated with water (paired t-test, *, p<0.05) while there was no significant effect of time on sugar accumulation in water (one-way Anova p=0.30) and in orthovanadate treatment (one-way Anova, p=0.22).

(C) Effect of vanadate on buffer pH. No differences are found between the two treatments; pH values do not change over the incubation period with vanadate while a one-way Anova test suggests differences between pH values for DI water over the time (p<0.05). Letters denote homogeneous groups based on Fisher LSD test (lower-case letter, differences between the two treatments; upper-case letter, differences among times). Data are mean values and bars are SE. Orthovanadate concentration used was 0.1mM which was added at time = 0 of the incubation.

Figure 7

Schematic illustration of proposed membrane transporter activity during onset of stress and recovery from stress. Results from former studies (Secchi et al., 2011, Secchi & Zwieniecki, 2012) are denoted as gray scale part of the figure. New aspect of related to the role of pH on the efflux of sugars to apoplast studied in this work is denoted by blue arrows and red text. For details of overall of scenario describing stem parenchyma cell activity, please refer to the text.

627 **Figure 1**

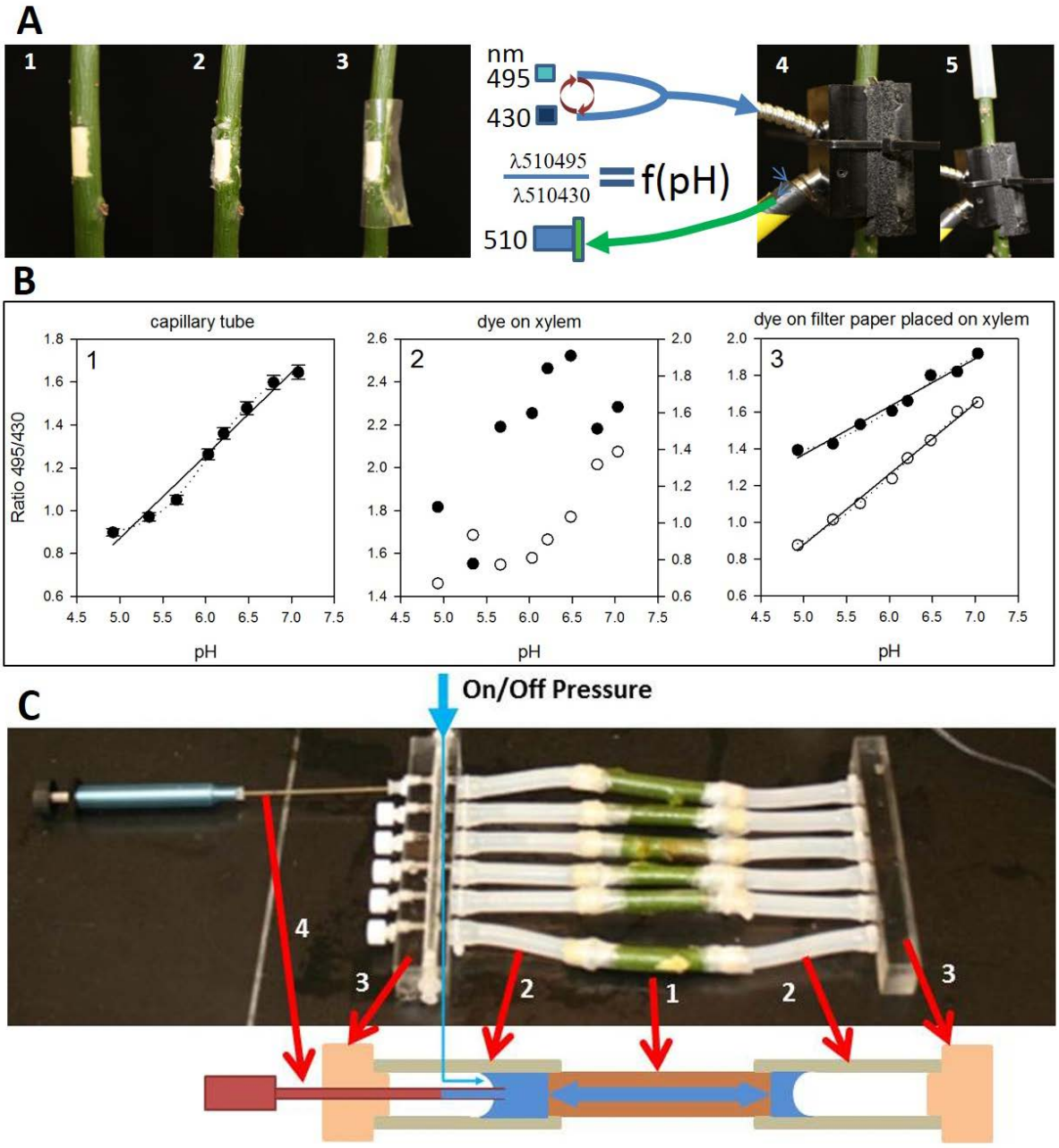

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Figure 2

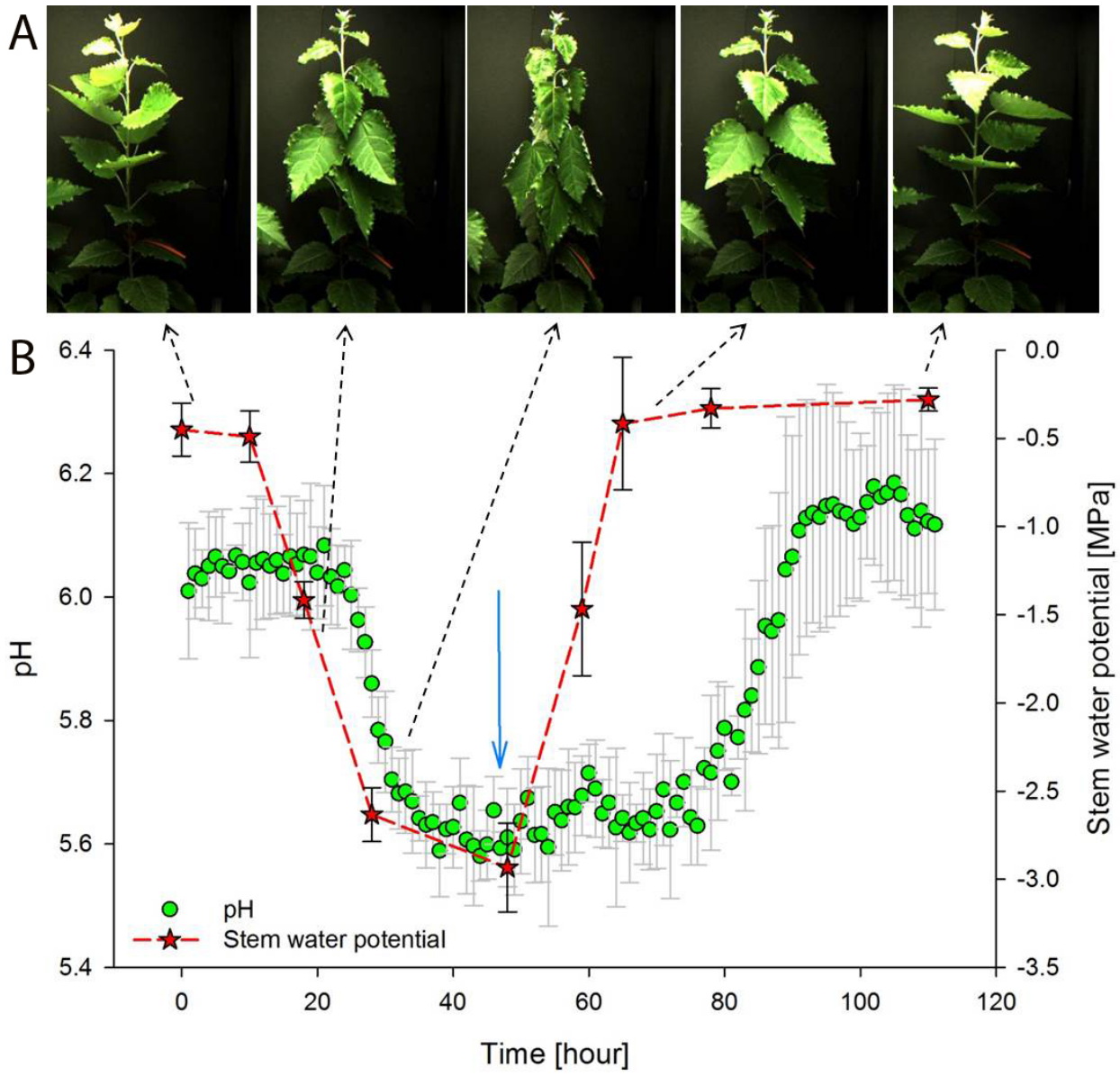
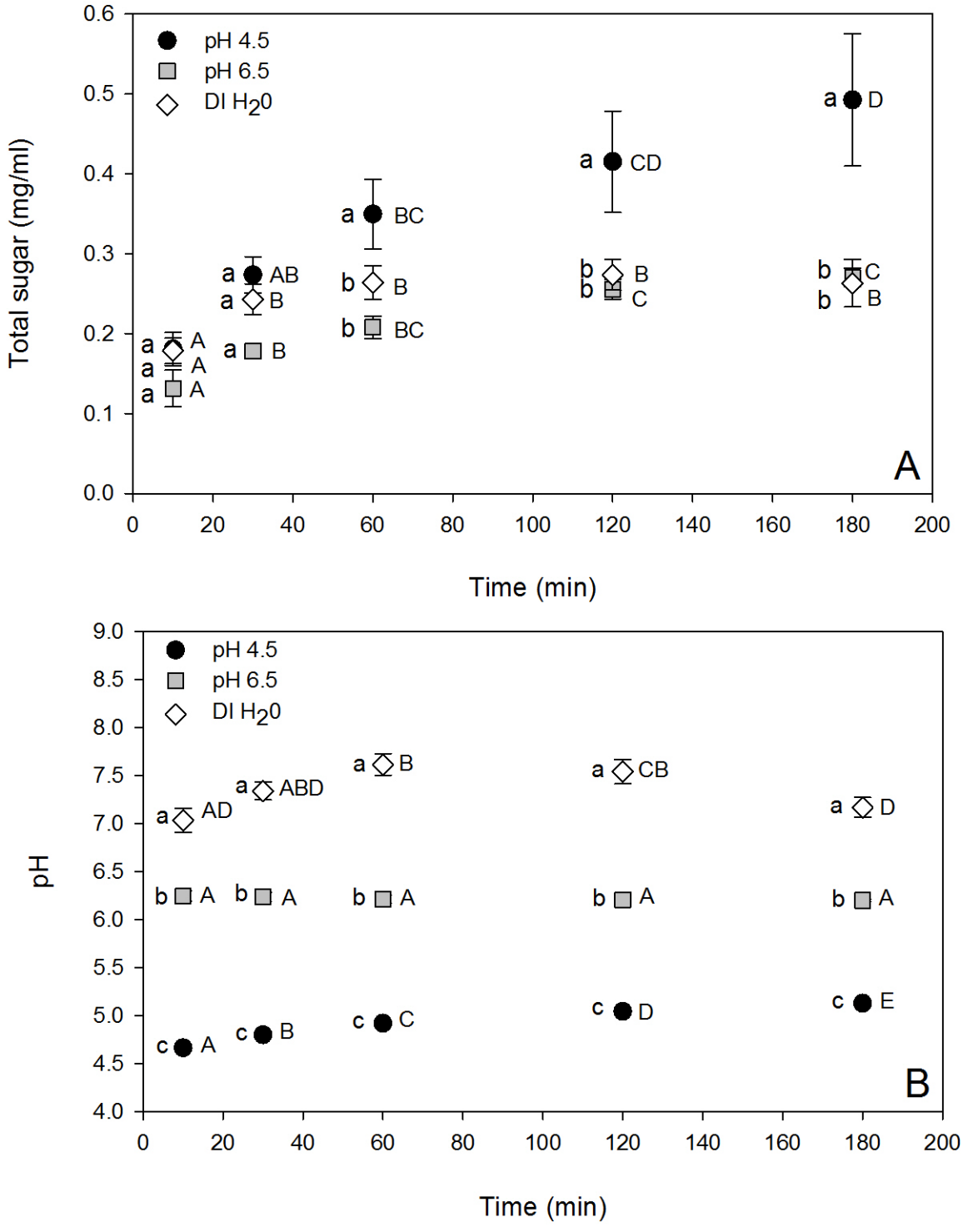
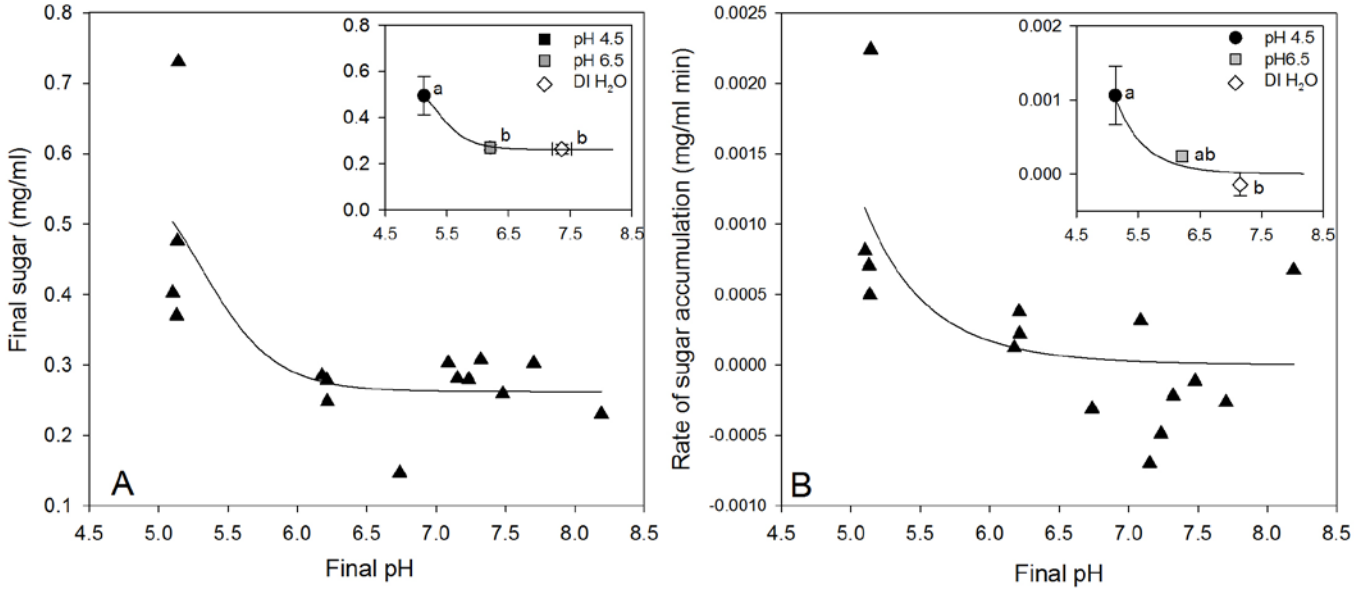

A
B
pH
Stem water potential [MPa]
Time [hour]
pH
Stem water potential
6.4
6.2
6.0
5.8
5.6
5.4
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
-3.0
-3.5
0
20
40
60
80
100
120

Figure 3



637 **Figure 4**



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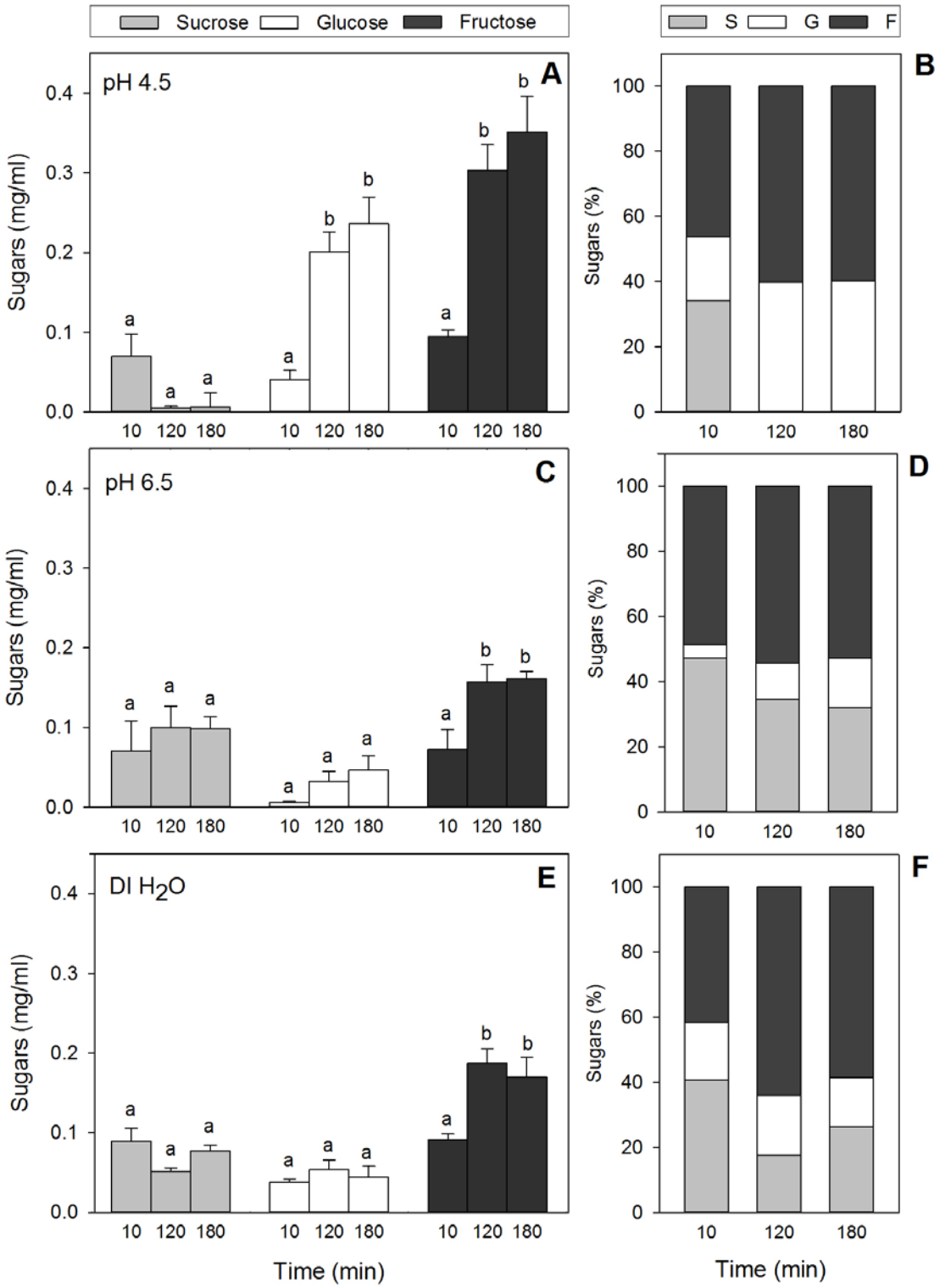
640 **Figure 5**



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643 **Figure 6**

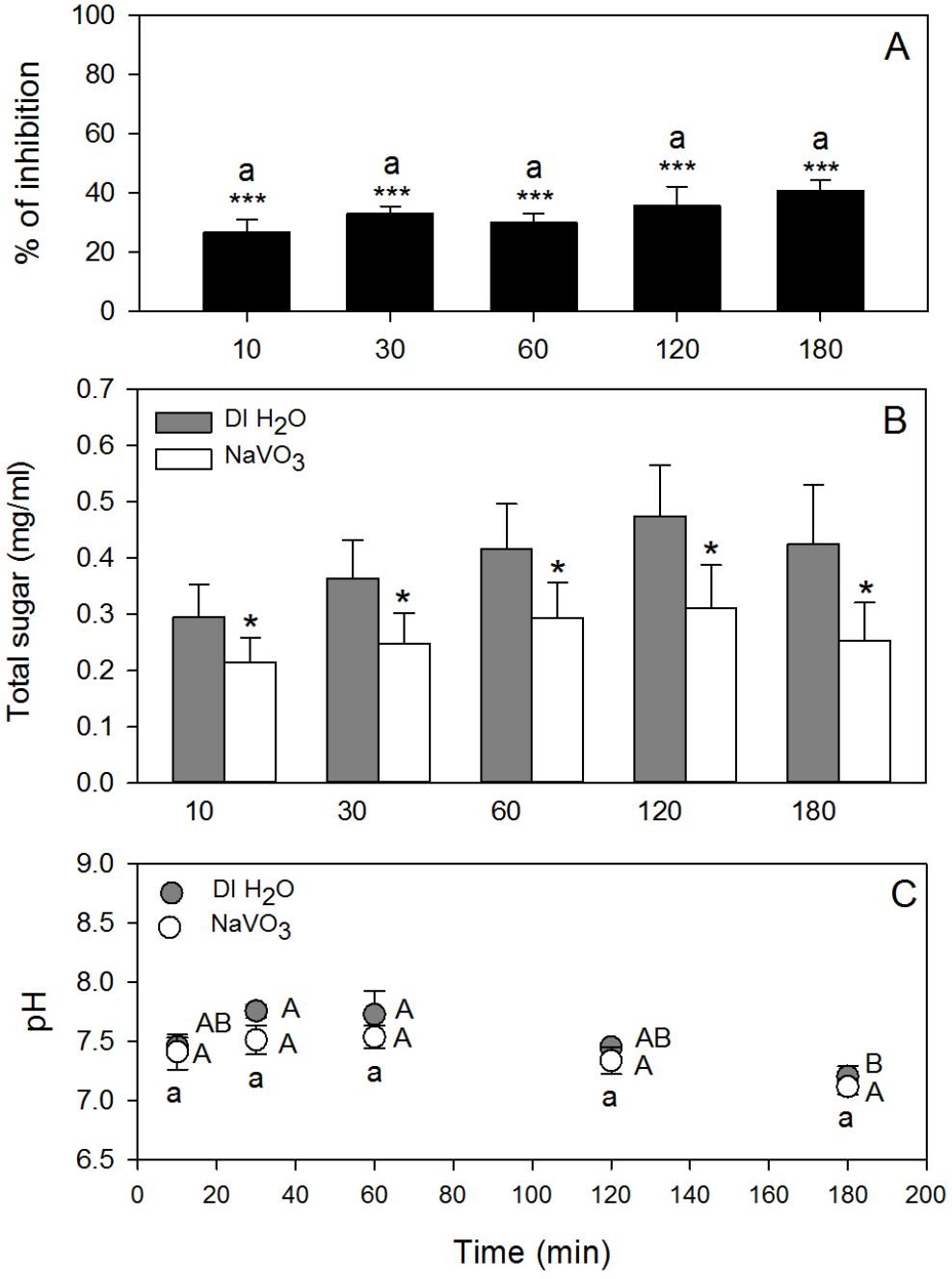

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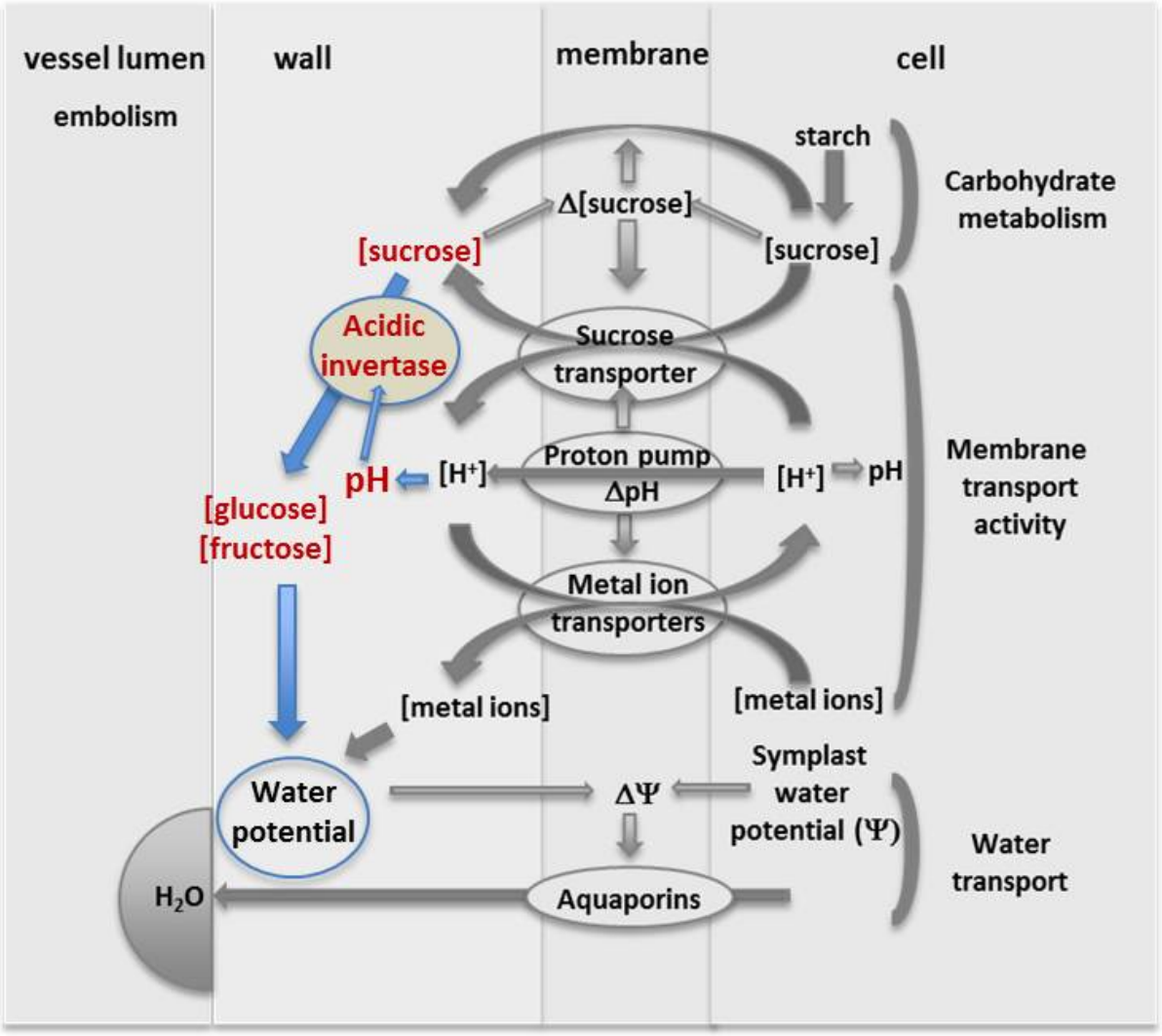
Figure 7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Symplast water potential (Ψ)
Water potential
ΔΨ
Water transport
H2O
Aquaporins