

# Uptake of Water via Branches Helps Timberline Conifers Refill Embolized Xylem in Late Winter<sup>1</sup>[C][W][OPEN]

Stefan Mayr\*, Peter Schmid, Joan Laur, Sabine Rosner, Katline Charra-Vaskou, Birgit Dämon, and Uwe G. Hacke

Department of Botany, University of Innsbruck, 6020 Innsbruck, Austria (S.M., P.S., B.D.); Department of Renewable Resources, University of Alberta, Edmonton, Alberta, Canada T6G 2E3 (J.L., U.G.H.); Institute of Botany, University of Natural Resources and Life Sciences, BOKU Vienna, 1180 Vienna, Austria (S.R.); and Institut National de la Recherche Agronomique, Unité Mixte de Recherche Physique et Physiologie Intégratives de l'Abre Fruitier et Forestier Site de Crouël, 63039 Clermont-Ferrand, France (K.C.-V.)

Xylem embolism is a limiting factor for woody species worldwide. Conifers at the alpine timberline are exposed to drought and freeze-thaw stress during winter, which induce potentially lethal embolism. Previous studies indicated that timberline trees survive by xylem refilling. In this study on *Picea abies*, refilling was monitored during winter and spring seasons and analyzed in the laboratory and in situ experiments, based on hydraulic, anatomical, and histochemical methods. Refilling started in late winter, when the soil was frozen and soil water not available for the trees. Xylem embolism caused up to  $86.2\% \pm 3.1\%$  loss of conductivity and was correlated with the ratio of closed pits. Refilling of xylem as well as recovery in shoot conductance started in February and corresponded with starch accumulation in secondary phloem and in the mesophyll of needles, where we also observed increasing aquaporin densities in the phloem and endodermis. This indicates that active, cellular processes play a role for refilling even under winter conditions. As demonstrated by our experiments, water for refilling was thereby taken up via the branches, likely by foliar water uptake. Our results suggest that refilling is based on water shifts to embolized tracheids via intact xylem, phloem, and parenchyma, whereby aquaporins reduce resistances along the symplastic pathway and aspirated pits facilitate isolation of refilling tracheids. Refilling must be taken into account as a key process in plant hydraulics and in estimating future effects of climate change on forests and alpine tree ecosystems.

Water transport in plant xylem is based on continuous water columns transmitting tension from leaves to roots and soil. This liquid continuum is metastable because cavitation can disrupt water columns and lead to embolism in the conduit network (Tyree and Zimmermann, 2002). During drought stress, increasing tension (i.e. decreasing water potential) causes air entry into xylem conduits via pits when critical thresholds in water potential ( $\Psi$ ) are reached. Hydraulic dysfunction may also be caused by freeze-thaw events, a common phenomenon in plants growing in seasonal or high elevation environments (Pittermann and Sperry, 2006). Bubbles formed during the freezing of xylem sap may expand as the sap melts and low  $\Psi$ s are re-established.

The biological significance of xylem embolism is that it blocks sap flow and impairs water supply of distal tissues. Over short periods of time, plants can control  $\Psi$  by stomatal adjustment of transpiration (Sperry and Pockman, 1993). Developmentally, plants cope with xylem tensions by investing in xylem structures that provide adequate safety from embolism (Hacke and Sperry, 2001; Sperry, 2003; Choat et al., 2012). When avoidance strategies fail (e.g. in the case of intense drought stress), repair strategies may prevent excessive shoot dieback. Repair of the hydraulic system may be based on the formation of new xylem or on refilling of dysfunctional xylem conduits. Several tree species can facilitate the latter by creation of positive pressures in the roots or the stem (Sperry et al., 1988b; Hacke and Sauter, 1996), but refilling has also been reported to take place at negative  $\Psi$  (Zwieniecki and Holbrook, 2009; Nardini et al., 2011; Brodersen and McElrone, 2013; Zwieniecki et al., 2013). Although the underlying mechanism is not yet understood, the following factors have been implicated to be involved (Holbrook and Zwieniecki, 1999; Hacke and Sperry, 2003; Zwieniecki and Holbrook, 2009; Nardini et al., 2011): isolation of refilling conduits from functional ones, a pathway and a driving force allowing water shifts into embolized conduits, a source of water increasing  $\Psi$  in conduits, as well as signal transduction networks initiating and integrating the process. At least some parts of this sequence are likely based on cellular activities such as

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\* Address correspondence to stefan.mayr@uibk.ac.at.

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the creation of a driving force by changes in osmotic potential, adjustment of pathway resistances via aquaporins, and cellular signaling. The isolation of conduits is an important prerequisite because water columns under tension, unless disconnected from the refilling conduit, would immediately suck out any water released to the conduit.

Vessel refilling in the absence of positive pressure has been reported in angiosperm species, whereby the xylem parenchyma and its interaction with the phloem were hypothesized to play a central role (Bucci et al., 2003; Brodersen et al., 2010; Nardini et al., 2011). Surprisingly, refilling was also observed in conifers, although their stem xylem contains a comparatively small amount of parenchyma tissue. For example, in *Pseudotsuga menziesii*, a percent loss of conductivity (PLC) of up to 60 in winter was followed by complete xylem repair (McCulloh et al., 2011).

Winter embolism was also found in conifers growing at high elevation. Sparks and Black (2000) found approximately 35 PLC in *Pinus albicaulis* and more than 25 PLC in *Larix lyallii* at the Rocky Mountains treeline. At the European alpine timberline, conifers can exhibit up to 100 PLC, induced by a combination of drought and freeze-thaw stress (Mayr et al., 2002, 2006a). These trees experienced frost drought, because frozen soil blocked water uptake for months. Moreover, the crown is exposed to numerous freeze-thaw events, with more than 100 frost cycles per winter, causing embolism even in conifers with small and resilient tracheids (Mayr et al., 2003a).

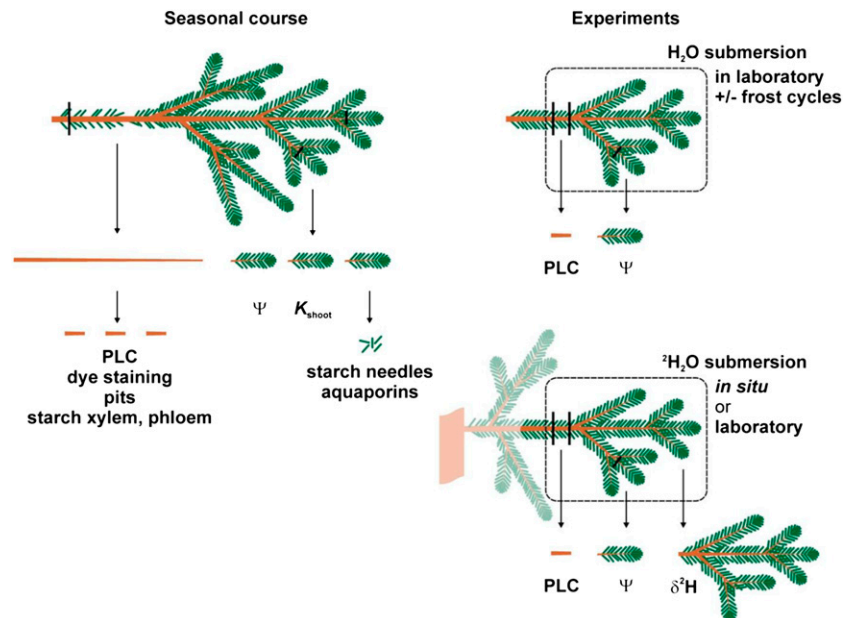
There are indications that these conifers survive winter embolism by recovery from hydraulic failure in late winter and spring. In branches of *Picea abies* trees growing along a transect up to the timberline, a significant increase in  $\Psi$  and hydraulic conductivity was

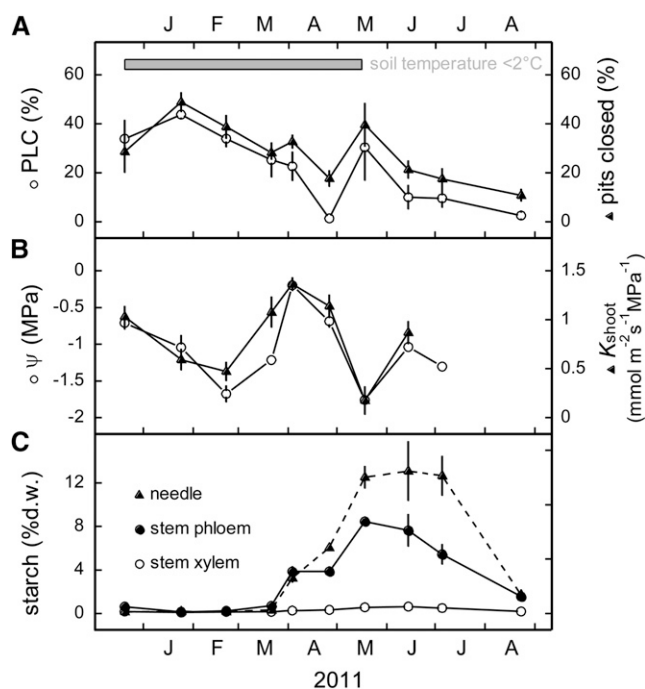
observed within 2 weeks in March (Mayr et al., 2002). Seasonal courses in *P. abies* and other conifers revealed a recovery process that was stepwise and lasted several weeks. It resulted in negligible PLC by April to June (Mayr et al., 2003b, 2006a). It has to be emphasized that the first steps of this recovery occurred when snow cover was still present and, consequently, uptake of soil water was blocked by ice barriers in the soil and/or stem base. This raises the question where the water required for the increase in  $\Psi$  and hydraulic conductivity might have come from.

Several studies demonstrated that plants can take up water not only via the root system but also via the leaves, under certain circumstances (Burgess and Dawson, 2004; Limm et al., 2009). In *Pinus contorta*, water uptake via branches during winter probably enabled an increase in  $\Psi$  and supported recovery from winter embolism (Sparks et al., 2001). The authors of this study suggested that the source of water was snow melting on branch surfaces. This process may also play a role for trees at the alpine timberline. Branches of evergreen conifers are often covered by snow, which melts in late winter, when the radiation becomes stronger, thereby intensively wetting branches. Foliar water uptake thus may enable an overall increase in  $\Psi$  during late winter; however, the question remains regarding whether it also allows refilling.

For refilling, water has to be shifted to embolized xylem sections. In conifer needles, water may reach the central cylinder via the apoplast or cellular pathways. It is known that aquaporins facilitate water flow between cells, allowing plants to modulate membrane resistance (Maurel et al., 2008; Gomes et al., 2009; Heinen et al., 2009). Accordingly, aquaporins could also be involved in facilitating radial water movement

**Figure 1.** Design of analysis in seasonal courses and experiments. For seasonal courses (left), branches were harvested in intervals. End segments were used for determination of  $\Psi$ , hydraulic conductance ( $K_{shoot}$ ), as well as analyses of starch and aquaporins in needles. The main stem was used for analyses of PLC and of conductive cross-sectional areas (dye staining), ratio of closed pits, and starch contents in xylem and phloem. In experiments (right), branches were harvested at the timberline and submersed in a water bath, before  $\Psi$  was measured on end twigs and PLC was measured in stem samples. Branches were also submersed in  $^2H_2O$ -labeled water in situ or in a water bath in the laboratory before  $\Psi$ , PLC, and  $\delta^2H$  of extracted xylem sap were determined. For details, see "Materials and Methods." [See online article for color version of this figure.]





**Figure 2.** Course of embolism, pit closure, water potential, shoot conductance, and starch content during winter and spring. A, PLC and the ratio of closed versus total number of pits. Duration of blocked water uptake (soil temperature below 2°C) is indicated by a gray bar. B,  $\Psi$  and hydraulic conductance in distal twigs ( $K_{shoot}$ ). C, Starch content (related to dry weight [d.w.]) is given for needles, stem phloem, and stem xylem. Mean  $\pm$  s.e. Months are indicated in respective order as follows: J, January; F, February; M, March; A, April; M, May; J, June; J, July; and A, August.

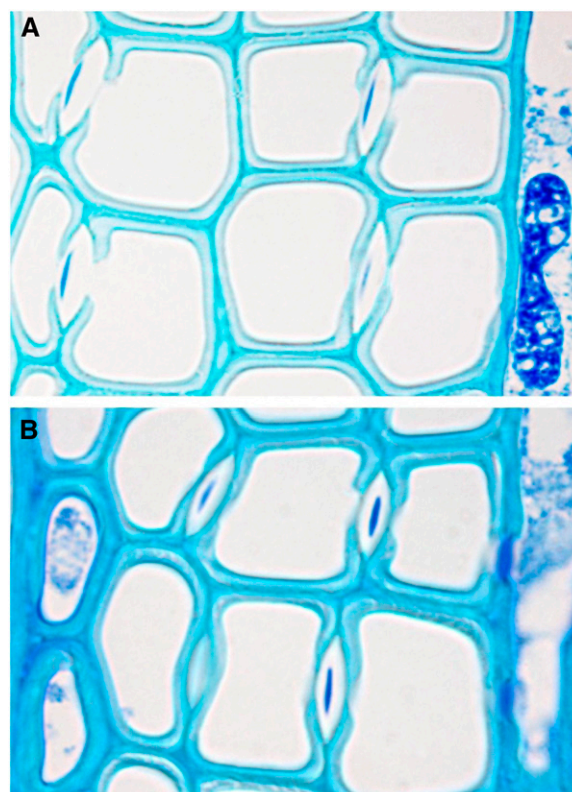
in needles. The driving force for water shifts may be created by osmotic adjustments as previously suggested (Holbrook and Zwieniecki, 1999; Hacke and Sperry, 2003; Zwieniecki and Holbrook, 2009; Nardini et al., 2011). Degradation of starch to osmotically active carbohydrates may enable local adjustments in  $\Psi$  to drive refilling of embolized conduits. However, it is unclear whether conditions in the winter at the alpine timberline allow cellular activities such as synthesis or modification of aquaporins, creation of osmotic gradients, and signaling (which is likely required to coordinate these processes). Even in late winter, nighttime temperatures frequently fall below the freezing point and tissues are exposed to high daily temperature fluctuations (Mayr et al., 2006b). Just the isolation of embolized from functional conduits, which is another prerequisite for refilling (see above), probably does not require metabolic activity. Conifer pits act like valves, which are passively closed by aspiration of the torus to the porus of the pit chamber (Pittermann et al., 2005; Hacke and Jansen, 2009; Plavcová et al., 2014). This mechanism might be especially advantageous to ensure isolation during refilling. To our knowledge, a correlation between PLC and pit closure has not been demonstrated up to now.

In this study, we analyzed the hydraulic recovery from winter embolism in *P. abies* trees growing at the alpine timberline. *P. abies* is one of the most economically important forest tree species in Europe. Using a combination of field measurements and laboratory experiments, we hypothesized that (1) hydraulic recovery of timberline conifers is based on refilling and not on the formation of new xylem, (2) isolation of embolized tracheids is achieved by reversible pit aspiration, (3) active, cellular processes take place in late winter, and (4) water uptake via the needles supports refilling. Despite low night temperatures and persisting snow cover, changes in aquaporin contents in the needle mesophyll and changes in starch pools were thus expected. Analyses were based on several hydraulic, anatomical, and histochemical methods.

## RESULTS

### Seasonal Course

Experiments were conducted as shown in Figure 1. Xylem embolism was highest in January ( $43.4 \pm 1.6$  PLC at January 25, 2011) and decreased during March and April to a PLC of  $1.0 \pm 0.6$ . In this period, water uptake from the soil was permanently blocked (Fig. 2A, gray horizontal bar). After a temporary increase of



**Figure 3.** Pits in stem xylem. Cross sections of earlywood tracheids in midwinter (A) and spring (B). Cells on the right are from ray parenchyma. [See online article for color version of this figure.]



PLC to  $30.0 \pm 13.5$  in May, a second recovery period in spring and summer resulted in a nearly complete reversal of embolism by August ( $2.2 \pm 1.6$  PLC). The ratio of closed pits showed a similar course (Fig. 2A). Nearly one-half of all intertracheid pits were closed in January and opened as PLC decreased (Fig. 3).

The  $\Psi$  decreased to  $-1.69 \pm 0.11$  MPa in February and then increased to  $-0.21 \pm 0.01$  MPa in April (Fig. 2B). During this period, PLC decreased, whereas a drop in  $\Psi$  to  $-1.77 \pm 0.02$  MPa in May corresponded with a temporary increase in PLC. Shoot conductance ( $K_{\text{shoot}}$ ) was highly variable (Fig. 2B) and closely tracked changes in  $\Psi$ . The maximum  $K_{\text{shoot}}$  ( $1.35 \pm 0.1$   $\text{mmol m}^{-2} \text{s}^{-1} \text{MPa}^{-1}$ ) was reached in April and the lowest values were reached in late February and May.

Starch contents (Fig. 2C) were highest in needles (maximum  $13.0\% \pm 2.7\%$  of dry weight at June 15, 2011) and lowest in the xylem (maximum  $0.6\% \pm 0.1\%$  of dry weight at June 15, 2011). Starch contents started to increase in early April and decreased in June/July. In August, starch contents were below 1.7% of dry weight in all tissues. According to determined starch concentrations, microscopic analysis revealed the highest starch grain densities in needles and phloem in May (Fig. 4, B and C).

Dye staining experiments (Fig. 4A) on stems revealed large areas of embolized xylem in midwinter (February 22, 2011) and decreasing dysfunctional portions during late winter and spring. In early summer (July 6, 2011), the complete cross section of branch stems, including all older year rings, was conductive.

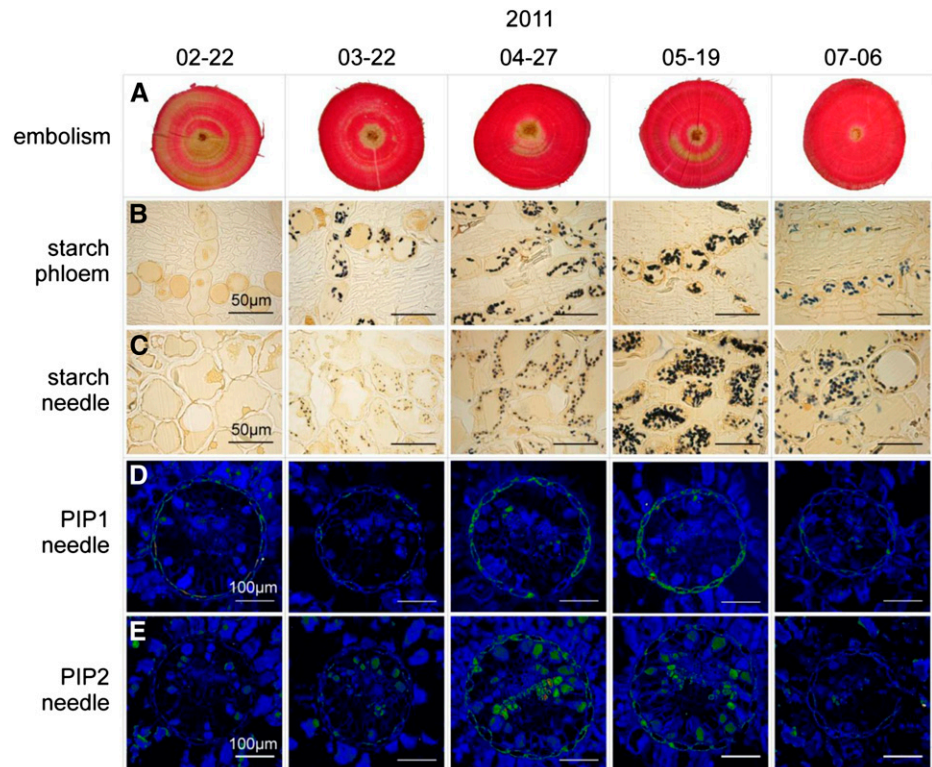
An increase in aquaporins (Fig. 4, D and E) in the endodermis and phloem cells of needles was observed from March 22, 2011 on and peaked in April and May. Plasma membrane intrinsic proteins (PIPs) of the subfamily PIP1 were highly abundant in endodermis cells, which contained Casparian bands (Fig. 5) in their radial cell walls. By contrast, PIPs of the subfamily PIP2 was predominantly present in the phloem cells of the central cylinder. Between May 19, 2011 and July 6, 2011, aquaporin contents decreased.

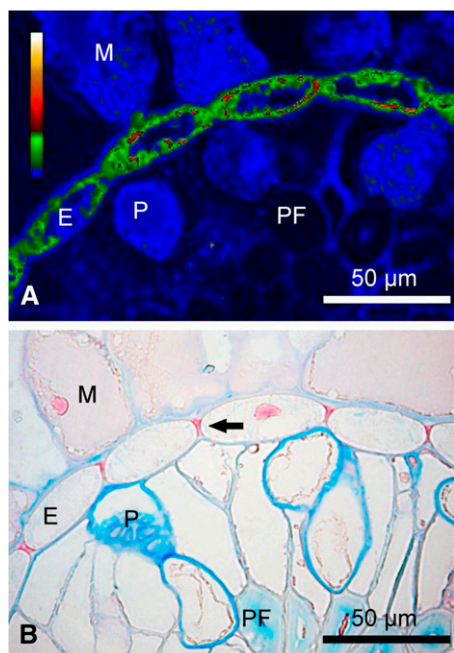
The seasonal course revealed correlations (Fig. 6) between PLC ( $r = 0.927$ ) and the ratio of closed pits and between  $\Psi$  and  $K_{\text{shoot}}$  ( $r = 0.889$ ). Less correspondence was observed between the dynamics of PLC and  $K_{\text{shoot}}$  ( $r = 0.485$ ). No correlation between starch contents and PLC or  $\Psi$  was observed over the season, but the onset of the starch increase in April corresponded with the highest reduction in PLC (Fig. 2). Similarly, PIP contents were highest during this period (Fig. 4).

### Refilling Experiments

In experiment 1, branches harvested at the timberline showed a  $\Psi$  of  $-3.58 \pm 0.24$  MPa and a PLC of  $86.2 \pm 3.1$  (Fig. 7A, d 0). Submersion of distal branch parts in water for 5 d caused a significant recovery of PLC to  $28.9 \pm 7.3$  and a recovery of  $\Psi$  to  $-1.16 \pm 0.19$  MPa (Fig. 7A, +H<sub>2</sub>O). Submersion and exposure to freeze-thaw cycles in parallel resulted in less complete yet significant recovery of PLC to  $64.9 \pm 2.0$  (Fig. 7A,

**Figure 4.** Structural and functional changes during winter and spring. A, Phloxine-B staining of branch xylem cross sections. Red areas indicate hydraulically intact regions, and uncolored areas are embolized. B and C, Starch granules stained with Lugol solution in branch phloem and needle mesophyll. D and E, Immunolocalization of PIP1 and PIP2 aquaporins in the transfusion tissue of needle cross sections. Intensity of labeling was encoded via a look-up table (see "Materials and Methods"; red, strong signal; black, background). Dates of sampling are given on the top.





**Figure 5.** Needle endodermis. A, Immunolabeling of PIP1 aquaporins in a needle cross section. Antibody signal intensities were encoded with a look-up table (see “Materials and Methods” as well as Fig. 4; red, strong signal; black, background). B, Staining of Casparian bands in the endodermis (arrow). E, Endodermis; M, mesophyll; P, parenchyma cell in transfusion tissue; PF, phloem fiber.

+H<sub>2</sub>O+FT). Control branches exposed to freeze-thaw cycles in the air showed no change in PLC (data not shown).

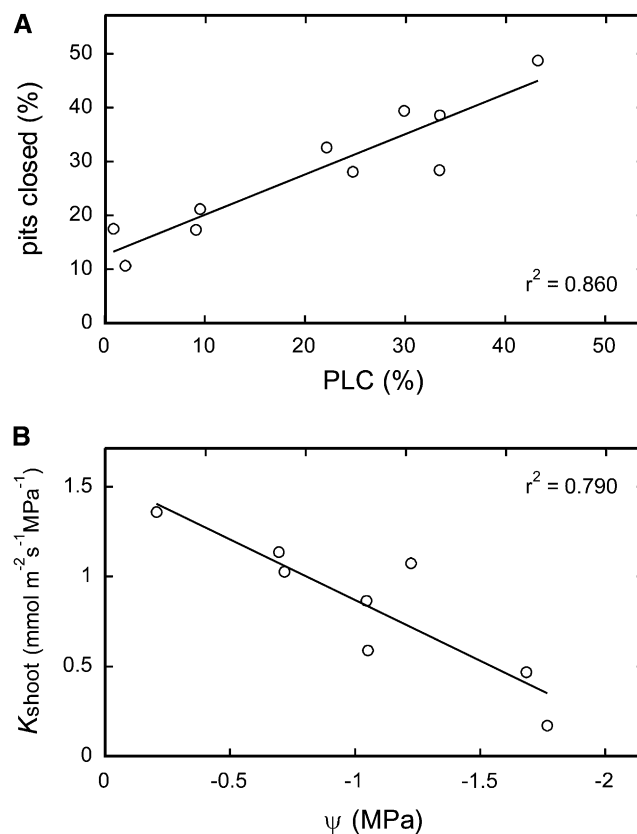
Experiment 2 showed that native PLC in branches collected at the timberline was  $64.3 \pm 6.6$  (Fig. 7B, d 0); the  $\Psi$  of branches was  $-0.84 \pm 0.05$  MPa. Exposure to water at the timberline for 21 d resulted in recovery of PLC to  $46.9 \pm 12.5$  ( $\Psi -0.24 \pm 0.06$  MPa). In the laboratory, PLC decreased to  $16.1 \pm 8.8$  ( $\Psi -0.04 \pm 0.02$  MPa) within 10 d (Fig. 7B). The  $\delta^2\text{H}$  in the sap of branches indicated that substantial amounts of water were taken up from the solution (Fig. 7C). At the timberline,  $\delta^2\text{H}$  of the xylem sap was  $54,500\text{‰} \pm 3,096\text{‰}$  compared with  $31,670\text{‰} \pm 3,180\text{‰}$  in the laboratory. The xylem sap of a control branch showed a  $\delta^2\text{H}$  of  $-38.1\text{‰}$ . In Figure 7C, values are given in relation to the measured  $\delta^2\text{H}$  of the labeled water (100% corresponding to  $92,000\text{‰} \delta^2\text{H}$ ). At the timberline, more than one-half of the extracted sap derived from external (labeled) water compared with about one-third in the laboratory experiment.

## DISCUSSION

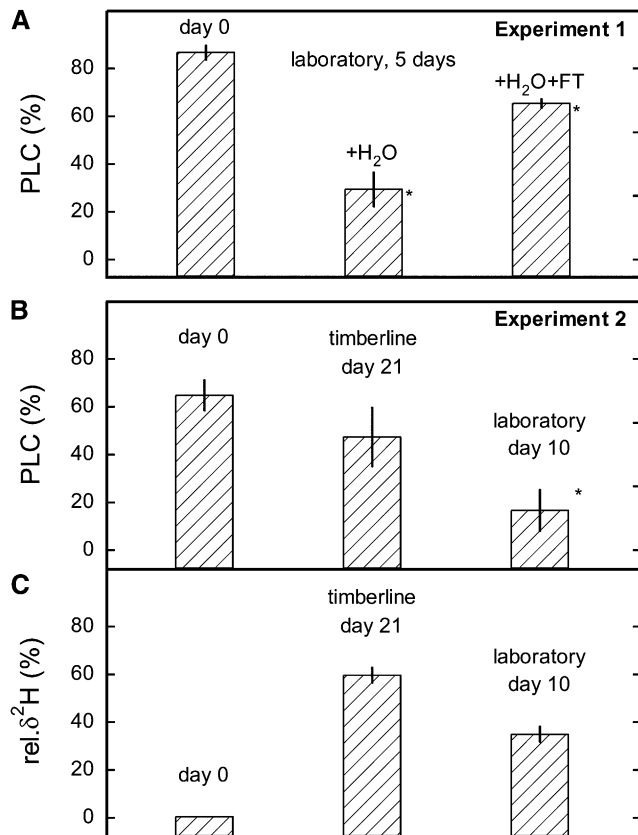
Conifers growing at the timberline can exhibit high embolism levels, as shown for *P. abies* in previous studies and this study. PLC in winter 2011 reached  $43.4 \pm 1.6$  (Fig. 2). PLCs in winters 2006 and 2008,

when experiments were performed, were  $86.2 \pm 3.1$  and  $64.3 \pm 6.6$ , respectively (Fig. 7). In accordance with previous reports on *Abies*, *Juniperus*, *Larix*, *Picea*, *Pinus*, *Pseudotsuga*, *Thuja*, and *Tsuga* spp. (Sperry et al., 1994; Sparks and Black, 2000; Mayr et al., 2002, 2003b, 2006a; McCulloh et al., 2011), the seasonal course showed a stepwise decrease in PLC that started as early as February (Fig. 2) while snow cover was still present and access to soil water was blocked by ice in the stem base and upper soil layers. This study focused on this hydraulic recovery, which enables timberline trees to restore hydraulic transport capacities before they enter the vegetation period.

In agreement with the hypotheses outlined above, our study provided the following insights into the recovery process in *P. abies*. First, the dye staining experiment (Fig. 4A) on stems showed that recovery was based on refilling of embolized tracheids and not on the formation of new xylem. Unstained areas within cross sections indicated large dysfunctional xylem regions during winter, whereas the entire xylem was conductive in the following growing season. A remarkable consequence is that the xylem of older year rings obviously contributes considerably to the hydraulic function of the stem although they passed numerous embolism/refilling cycles. The tracheids of



**Figure 6.** Correlation analysis. Correlation of the ratio of closed pits versus PLC and  $K_{\text{shoot}}$  versus  $\Psi$ .



**Figure 7.** Experimental analysis of water uptake via the branch surface. A, PLC of stem xylem from branches harvested at the timberline. PLC before (d 0) and after 5 d of submersion in water (+H<sub>2</sub>O) is given. In addition, branches were exposed to 10 freeze-thaw cycles during submersion (+H<sub>2</sub>O+FT). B, PLC of stem xylem from branches harvested at the timberline. Branches were either exposed to <sup>2</sup>H-labeled water in situ at the timberline for 21 d or cut and submersed in labeled water in the laboratory for 10 d. C,  $\delta^2\text{H}$  of xylem sap extracted from stems of branches submersed in situ at the timberline or in the laboratory [see (B)]. Day 0 shows  $\delta^2\text{H}$  of a native branch.  $\delta^2\text{H}$  is given as percentage of  $\delta^2\text{H}$  of the labeled submersion solution. Mean  $\pm$  SE. Asterisks indicate significant differences.

studied conifers thus did not suffer from cavitation fatigue (Hacke et al., 2001), which corresponds to findings of Johnson et al. (2011, 2012).

Second, torus-margo pits of conifers with their valve-like closing mechanism (Hacke et al., 2004; Pittermann et al., 2005; Fig. 3), are likely responsible for separating air and water phases within the xylem, and thus for the required isolation of embolized conduits during the refilling process. As shown in Figure 2, the ratio of aspirated pits closely matched the course of PLC, indicating that all embolized tracheids exhibited closed pits. Accordingly, a high correlation between PLC and the ratio of closed pits was observed (Fig. 6A). Conifer pits thus provide an efficient mechanism to ensure the necessary isolation of dysfunctional tracheids from adjacent, intact xylem sections (Zwieniecki and Holbrook, 2009). At the timberline, winter embolism is induced not only by frost drought but also by

freeze-thaw events, where conduits cavitate independently and pits therefore may isolate conduit by conduit.

Third, when refilling started, several cellular processes were active. Starch contents increased from the end of February onward and peaked during the time of highest refilling activities (Figs. 2 and 4, B and C). According to refilling models for angiosperms (Nardini et al., 2011), starch may be degraded to osmotically active sugars, which are released into embolized conduits thereby creating a driving force for water inflow (Bucci et al., 2003; Secchi and Zwieniecki, 2012). Observed starch accumulation in phloem and needle tissues may thus be required to provide sufficient osmotica for local changes in  $\Psi$ . Interestingly, we found no significant seasonal changes in Glc or Fru (data not shown). Because osmotica have to be released only into refilling tracheids, variations in sugar concentrations probably occur on a small spatial scale and thus cannot be detected by analysis of entire tissue samples. In *Populus*, a special method enabled the analysis of sugar contents in embolized and intact vessels (Secchi and Zwieniecki, 2012).

As hypothesized, we found increasing amounts of PIP1 and PIP2 proteins in the needle endodermis and in phloem cells beginning in March and peaking at the time of highest refilling activities (Fig. 4, D and E). PIP1 was highly abundant in endodermis cells, which contained Casparian bands in their radial cell walls (Fig. 5). This is consistent with findings in *Picea glauca*, which showed similar dynamic and spatial patterns in needle PIP contents during recovery after a drought period (J. Laur and U.G. Hacke, unpublished data). Aquaporins may be important for directed water shifts because they reduce transport resistances of symplastic pathways. The transfusion tissue and the endodermis thus may play a role not only in phloem loading and water transport to the mesophyll (Liesche et al., 2011), but also in reverse water movements to embolized tracheids during refilling. This plays an important role in the case of foliar water uptake.

Fourth, as mentioned above, melting snow may be a source of water taken up by the branches. Experiments with labeled water proved that a relevant amount of water can be taken up via the branch surface (Fig. 7C). According to the  $\delta^2\text{H}$  analyses, about one-third of the xylem sap derived from external water in the laboratory experiment. At the alpine timberline, even more than one-half was taken up via the branch surface. Katz et al. (1989) previously reported water uptake via the surface of *P. abies* twigs. Their experiments with fluorescent dyes revealed pathways for the absorption of water and solutes through the twig bark and ray tissue. An uptake of water via the needles is also likely, whereby partly opened stomata (Mayr et al., 2012) and the cuticle provide possible points of entry. It is known that the cuticle of timberline conifers can show insufficient maturation (Michaelis, 1934a, 1934b) and that the needle surface is often damaged by ice blast (Hadley and Smith, 1989; Gardingen et al., 1991). Accordingly, a

high cuticular conductance was reported for *P. abies* at high elevation (Anfodillo et al., 2002; Mayr et al., 2003b), which may amplify drought stress on the one hand, but facilitate water uptake on the other. Water uptake via the needles also explains differences in PLC of proximal and distal branch sections (Supplemental Fig. S1): Fluctuations in PLC were more pronounced in distal branch sections (where most of the needles and the largest snow loads are situated) leading to a faster decrease in PLC during the recovery phases in March and from mid-April. We also found that  $K_{\text{shoot}}$  recovered in late winter, whereby a close correlation with  $\Psi$  was observed (Figs. 2B and 6B). Several causes for declines in needle conductance have been discussed, including cavitation of needle xylem (Johnson et al., 2012). Accordingly, the recovery in  $K_{\text{shoot}}$  observed between February and March and again in May/June, may have been caused by reversible changes in the extravascular pathway, refilling of embolized tracheids, or a combination of both.

Laboratory experiments demonstrated that uptake of water corresponded with refilling activities in stems, as branches submersed in water, showed a 57.3 and 48.2 reduction of PLC (Fig. 7, A and B). Additional exposure to freeze-thaw stress during submersion led to a reduced recovery, because branches were exposed to fluid water only half of the time. At the timberline, in situ submersion of branches resulted in a 17.4 reduction of PLC (Fig. 7B). This is less than what was observed in laboratory experiments and may be explained by overall lower and fluctuating temperatures (limiting cellular activities), and the connection of refilling branches to crown parts with lower  $\Psi$  (so that water taken up was not only used for refilling within the sample branch). Water uptake from melting snow was also suggested to support embolism repair in *P. contorta* (Sparks et al., 2001), and a recent experiment with potted *P. glauca* plants (J. Laur and U.G. Hacke, unpublished data) demonstrated water uptake via the needles and subsequent xylem refilling.

Building on what has been suggested for angiosperms (Zwieniecki and Holbrook, 2009; Nardini et al., 2011; Johnson et al., 2012), we offer the following model to explain the observed refilling in timberline trees. In late winter, when air temperatures rise, refilling is induced and driven by metabolic activity of tissues in the crown. Sugar transport via the phloem enables building up of starch depots in stem and needle parenchyma. Part of the starch is degraded, and sugars are released into water films of embolized tracheids, which are isolated from functional conduits by aspirated pits. The decrease in  $\Psi$  causes water shifts into embolized tracheids and the dissolution of gas. Water required for filling tracheids is taken up via the needle surface. Following the  $\Psi$  gradient, water has to pass the endodermis on its way to the xylem and phloem of the central cylinder, which enables control of water uptake. Water then flows to dysfunctional xylem sections and reaches refilling tracheids via ray parenchyma (Almeida-Rodriguez and Hacke, 2012; Barnard

et al., 2013). Which signals trigger sugar release and aquaporin activity to direct water specifically to embolized conduits remains a fascinating question.

Refilling is a crucial aspect in the water relations of alpine timberline trees; the observed PLC would be lethal without repair. It is remarkable that repair mechanisms work under winter conditions and that physiologically relevant amounts of water can be taken up via the branch surface. A better understanding of this process will be fundamental for estimating future effects of climate change on tree ecosystems (Zhang et al., 2007; Allen et al., 2010) and especially on forests of the sensitive alpine regions.

## MATERIALS AND METHODS

### Plant Material

This study was performed on *Picea abies* trees growing at an elevation of 2035 m at Birgitz Köpfl in Tyrol, Austria (latitude, 47°11'N; longitude, 11°19'E). Measurements were made on sun-exposed branches of 2- to 4-m tall, single standing specimens in a northwest-exposed slope. We used branches at breast height with a length of up to 1 m for analysis in a seasonal course and up to 0.5 m in experiments. For logistic reasons, samples harvested at the timberline were sometimes stored at -20°C until measurements. When samples had to be transported between involved institutions, cooling boxes with dry ice were used.

### Seasonal Course

From December 2010 to August 2011, the study site was visited in approximately 4-week intervals and at least three branches (of different, randomly chosen trees) were harvested for measurements (Fig. 1). First, end twigs (approximately 10 cm) of selected branches were sampled and enclosed in plastic bags for transport to the laboratory and analysis of the following parameters ( $n = 3$ ):  $\Psi$ ,  $K_{\text{shoot}}$ , needle starch content, and immunolocalization of aquaporins. Second, the branches were cut and enclosed in plastic bags. After transport to the laboratory, these samples were recut under water to remove artificial embolism and to gradually release tension. Main stems were used for analysis of the following parameters ( $n = 3$ ): PLC, conductive xylem area (via dye staining), ratio of closed pits, and xylem and phloem starch content. Soil and xylem temperatures at the trunk base were monitored with a data logger (Minikin; EMS).

A second seasonal course (February to May 2004) is presented in Supplemental Figure S1, in which  $\Psi$  and PLC of proximal and distal branch sections were compared.

### Refilling Experiments

Two experiments to demonstrate and analyze refilling in situ as well as under laboratory conditions were performed (Fig. 1).

In experiment 1, embolized branches were harvested at the timberline and transported to the laboratory (February 22, 2006). A first set of branches ( $n = 4$ ) was used to determine native PLC ( $86.2 \pm 3.1$ ) and  $\Psi$  ( $-3.6 \pm 0.2$  MPa). A second set of branches was submersed in a tap water-filled tray for 5 d, whereby the proximal section (5 cm at minimum) of the branch stems was situated outside the water tray to avoid any water uptake via the cut surface of the main stem. A part of these samples ( $n = 4$ ) was kept at a water temperature of 15°C during the experiment. The other part ( $n = 4$ ) was exposed to 10 freeze-thaw cycles to simulate temperature fluctuations at the alpine timberline. Therefore, the water tray with submersed branches was positioned in a temperature chamber (MK53; Binder), which ran repeated cycles of the following temperature course: 4 h at +15°C, 2 h of cooling to -10°C, 4 h at -10°C, and 2 h of heating to 15°C during. Control branches ( $n = 5$ ) were wrapped in plastic bags and kept at 15°C for 5 d without submersion. After treatments (5 d), PLC and  $\Psi$  of all branches were determined.

Experiment 2 was made on embolized branches in situ and on cut branches transported to the laboratory (March 11, 2008). A first set of branches ( $n = 5$ )



was cut and used to determine native PLC ( $64.3 \pm 6.6$ ) and  $\Psi$  ( $-0.84 \pm 0.1$  MPa). A second set of branches ( $n = 5$ ) was bagged in situ and bags were filled with  $^2\text{H}$ -labeled water (about 2%  $^2\text{H}_2\text{O}$  [v/v]). These branches were cut after 21 d for analyses. A third set of branches ( $n = 5$ ) was transported to the laboratory and the distal end (approximately 30 cm) was submerged in a water-filled tray as described for experiment 1. In contrast with experiment 1, branches were submersed for 10 d and the tray was filled with labeled water (about 2%  $^2\text{H}_2\text{O}$  [v/v]). After treatments, PLC and  $\Psi$  of all branches were determined. Furthermore, xylem sap was extracted and  $\delta^2\text{H}$  determined from stems of branches submersed in situ ( $n = 4$ ) and in the laboratory ( $n = 3$ ).

## Water Potential

For  $\Psi$  determination, end twigs (approximately 10 cm) were measured with a Scholander apparatus (model 1000; PMS). For seasonal courses, three end twigs were measured and  $\Psi$  was averaged per branch. In experiments, only one end twig per branch was measured, because smaller branches were used.

## PLC

PLC was quantified with a Xyl'em system (Bronkhorst) on three to four subsamples (seasonal course) or one to six subsamples (experiments, number of subsamples limited by the size of the main stem) and averaged per branch. Stem segments, approximately 4 cm in length, were cut under water and the PLC was determined by measuring the increase in hydraulic conductivity after removal of xylem embolism by repeated high-pressure flushes (Sperry et al., 1988a). Sample preparation and measurement procedure followed a previously described protocol (Mayr et al., 2006a): Flushing (at 80 kPa) and conductivity measurements (at 4 kPa) were done with distilled, filtered (0.22  $\mu\text{m}$ ) and degassed water containing 0.005% (v/v) Micropur (Katadyn Products) to prevent microbial growth. Flushing was repeated until measurements showed no further increase in conductivity. The loss of conductivity was calculated as  $\text{PLC} = (1 - K_{\text{min}}/K_{\text{max}}) \times 100$ , where  $K_{\text{min}}$  is the initial conductivity and  $K_{\text{max}}$  is the maximal conductivity.

## Dye Staining

Fresh stem segments, approximately 5 cm in length, were cut under water and connected to a reservoir with 2% (w/v) Phloxine-B solution (Sigma Chemicals). After staining for several minutes, stems were cut in the center and photos of cross sections taken. Stained areas indicated functional xylem, while embolism blocked the flow of staining solution and resulted in unstained areas.

## Shoot Conductance

Rehydration kinetics were measured on end twigs (3 to 5 cm) as previously described (Brodribb and Holbrook, 2003). First, the capacitance ( $\text{mol m}^{-2} \text{MPa}^{-1}$ ) was quantified by dehydration and repeated measurements of weight (ME2355; Sartorius) and corresponding  $\Psi$  (see above).  $\Psi$  was plotted versus the relative water content, which was calculated from the corresponding weight and the saturated and dry weight of shoots. Capacitance in the  $\Psi$  range above the turgor loss point (0 to  $-2.8$  MPa) was  $0.276 \text{ mol m}^{-2} \text{MPa}^{-1}$ . It was determined at the beginning of 2011 and assumed to be constant throughout the season because previous measurements indicated no relevant change in  $\Psi$  versus relative water content relations during winter and spring (data not shown). Second, branches dehydrated to  $-2.8$  MPa at maximum were stored for equilibration at  $5^\circ\text{C}$  in plastic bags (approximately 1 h), before  $\Psi$  was determined ( $\Psi_1$ ). End twigs were cut under water and the basal end was placed in a tray with distilled water. After rehydration (5 to 400 s),  $\Psi$  of samples was determined ( $\Psi_2$ ).  $K_{\text{shoot}}$  ( $\text{mol m}^{-2} \text{s}^{-1} \text{MPa}^{-1}$ ) was calculated as  $K_{\text{shoot}} = C \times \ln(\Psi_1 / \Psi_2) \times t^{-1}$ , where  $C$  is capacitance and  $t$  is the time span (s). Test measurements (at different temperatures, repeated trimming of samples) proved that resin did not affect  $K_{\text{shoot}}$  determination (data not shown).

## Anatomical Analysis

Fresh sapwood specimens and needles were fixed in formalin-acetic acid-alcohol for at least 24 h. The fixed material was dehydrated in ethanol and embedded in Technovit 7100 (Heraeus Kulzer). Semithin transverse sections

(1 to 2  $\mu\text{m}$ ) were made on a rotary microtome (RM2235; Leica). Sections were stained either with toluidine blue and Safranin-Astra blue dye (Merck) and were mounted in Euparal (Carl Roth). Safranin-Astra blue dye stains lignified structures in bright magenta, nonlignified cell walls were stained blue. Recipes for the fixation medium and staining solutions can be found in Gerlach (1984). Staining of starch grains was performed with Lugol's solution (Fluka; Sigma-Aldrich).

Bordered pits were observed with a Leica DM4000 M microscope equipped with a digital camera (DFC320 R2; Leica). Pits were classified as closed if the pit membrane was aspirated or as open if the pit membrane was positioned in the middle of the pit chamber. Toluidine blue stains the torus of the pit in dark purple, which gives a strong contrast to the light blue-stained pit chamber walls and secondary cell walls of the tracheids (Rosner et al., 2010). Classification of pit aspiration was done separately for each of the latest three annual rings within a tangential window of 2 to 3 mm on two to four specimens from each branch.

## Starch Content

Dried and finely ground plant material was incubated two times with 80% (v/v) ethanol at  $75^\circ\text{C}$  with polyvinylpyrrolidone 40 (Sigma Chemicals) added. The supernatants containing the low  $M_n$  sugars were collected, the solvent was evaporated, and the residue was dissolved in citrate buffer, pH 4.0. After dissociating the Suc with invertase ( $\beta$ -fructosidase), the content of Glc was determined photometrically (Bauer et al., 2000). Fru was converted to Glc with isomerase (phosphoglucose isomerase) and quantified using a standard test (Enzytec E1247; R-Biopharm) measuring the absorption of NADPH (Bergmeyer and Bernt, 1974) at 340 nm. The precipitate containing the starch was incubated with sodium hydroxide (0.5 N, 1 h at  $60^\circ\text{C}$ ), neutralized with hydrochloric acid, treated with amylase (amylglucosidase) to break down the starch to Glc, and measured as described (Enzytec E1210; R-Biopharm).

## Immuno-localization

Needle samples were fixed in paraformaldehyde medium (4% [v/v] paraformaldehyde, 0.25% [v/v] glutaraldehyde in phosphate-buffered saline [PBS]) under vacuum for 2 h, processed, and embedded in paraffin as previously described (Sutton et al., 2007). Cross sections (8  $\mu\text{m}$ ) were prepared using a microtome, mounted on slides, and dried overnight at  $37^\circ\text{C}$ . The next day, sections were dewaxed twice for 10 min each in Histochoice (Sigma-Aldrich), rehydrated through an ethanol series, rinsed three times for 10 min in PBS, and incubated for 45 min in a blocking solution (1.5% [w/v] Gly, 5% [w/v] bovine serum albumin, 0.1% [v/v] Tween 20 in PBS) as previously described (Gong et al., 2006). Primary antibodies against the first 42 N-terminal amino acids of AtPIP1;3 (Kammerloher et al., 1994; 5'-M EGKEEDVRVGANKFPERQPIGTSAQSDKDYKEPPPAPFFEP-3') and the highly conserved 10 amino acids of the C-terminal of PIP2 aquaporins (similar to Daniels et al., 1994; 5'-KALGSFRSNP-3') were included in this study. Two secondary antibodies preabsorbed with plant tissue extract, DyLight 549 conjugated rabbit anti-chicken antibody (Fisher Scientific) and HiLyte Fluor 555 (AnaSpec) conjugated rabbit anti-mouse antibody, were applied for 2 h at  $37^\circ\text{C}$  as previously described (Gong et al., 2006). Slides were rinsed several times and coverslipped with a synthetic resin (Permount; Fisher Scientific). Controls with no primary and/or secondary antibody were also prepared.

Samples were examined on a TCS SP5 confocal microscope (Leica). Secondary antibodies were excited with the 543 nm line of a HeNe laser while the emission was detected in the corresponding range for each secondary antibody according to the manufacturer's specifications. For each primary antibody assay, all images were collected with Leica LAS AF 2.4.1 software at identical settings and processed using Fiji (Schindelin et al., 2012). From 8-bit images, the signal intensity values were displayed with a look-up table, in which blue was used to encode tissue background, and green, red, and yellow to encode increasing signal intensity.

## $\delta^2\text{H}$ Analysis

Branches submersed in  $^2\text{H}_2\text{O}$ -labeled solution in situ or in the laboratory were carefully dried with paper towel and the basal 5 cm was debarked. The distal parts (up to 30 cm) of branches and of control branches were sealed in a Scholander apparatus (see above). The pressure was slowly raised to 4 MPa and the sap dripping out of the basal cross section was collected in a 10-ml test tube. Therefore, the apparatus was mounted top-down with a tripod. After



5 to 10 min of sap collection, the tube was closed and immediately stored at  $-80^{\circ}\text{C}$ .

For determination of  $^2\text{H}$  contents, sap samples were sent to the Austrian Institute of Technology. Analyses were performed with an isotope mass spectrometer (ThermoFinnigan Delta plus xp; Fisher Scientific) with an automated thermal combustion elemental analyzer. Based on the Vienna Standard Mean Ocean Water standard,  $\delta^2\text{H}$  values of extracted sap and of the  $^2\text{H}_2\text{O}$ -labeled solution were calculated.  $\delta^2\text{H}$  of sap samples then were related to  $\delta^2\text{H}$  of the labeled solution, which was set to 100%. It has to be considered that  $\delta^2\text{H}$  analysis allows us to verify water uptake, whereas the quantity of the uptake can only be estimated. Water taken up may be distributed to branch sections situated outside the labeled solution and the  $\delta^2\text{H}$  composition of the xylem sap and its contents is unknown.

## Statistical Analysis

Values were averaged per branch, which is the statistical unit throughout the study, and are given as the mean  $\pm$  SE. Differences at  $P \leq 0.05$  were tested with the Student's *t* test after testing for Gaussian distribution (Kolmogorov-Smirnov test) and homogeneity of variances (Levene test). Correlations were tested with Pearson's coefficient at  $P \leq 0.05$ .

## Supplemental Data

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Embolism and water potential in different sections of branches during late winter.

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