Title: Visualization of seasonal shifts of phosphorus re-translocation and overwintering recycling in a shortened annual cycle system of the deciduous poplar tree (*Populus alba* L.).

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Summary

(1) the research conducted, including the rationale

Phosphorus (P) is an essential macronutrient for plant growth. In deciduous trees, P is remobilized from senescing leaves and stored in perennial tissues during winter for further growth. To clarify how deciduous trees utilize the internal P through a year, seasonal re-translocation routes and the reuse of P over winter were investigated.

(2) methods

We analyzed the seasonal re-translocation and accumulation of P in poplar trees (*Populus alba*. L) cultivated under “a shortened annual cycle system”, which mimicked seasonal phenology in a laboratory. The real-time radioisotope imaging and the macro- and micro-autoradiography with ^{32}P and ^{33}P were used to reveal the organ and tissue level P allocation.

(3) key results

The direction of phosphate re-translocation changed seasonally. In the growing season, P was mainly re-translocated from a leaf to upper parts via phloem. During senescence, P was re-translocated to lower perennial parts, and also to inner xylem parenchyma cells. Phloem-xylem exchange of re-translocated P occurred in the stem. After bud burst in spring, stored P was re-translocated to the whole plant and mainly accumulated in new shoots.

(4) the main conclusion, including the key points of discussion

Poplar trees change the routes of P re-translocation longitudinally and radially depending on the season, and recycle internal P throughout the year.

57 **Key words**

58 Autoradiography • Deciduous tree • Phosphorus • *Populus alba* • Seasonal re-

59 translocation

60

Introduction

Phosphorus (P) is an essential macronutrient for plant growth, but its availability in soil is quite limited (Schachtman *et al.*, 1998). To utilize the precious P, plants have various strategies for the uptake, translocation, storage and recycling of P, etc. Re-translocation of P is a strategy for its internal recycling *in planta*. P is re-translocated from source tissues (e.g. old leaves) to sink tissues (e.g. young leaves, shoot apex) as phosphate (Biddulph *et al.*, 1958b; Mimura *et al.*, 1996; Himmelblau & Amasino, 2001). It is supposed that such re-translocation of phosphate between tissues occurs via the phloem. Both downward and upward P re-translocation via the phloem from leaves, and the lateral movement from the phloem to xylem have been demonstrated using microradiographic methods in an herbaceous plant, red kidney bean (Biddulph, 1956; Biddulph *et al.*, 1958a).

In deciduous woody plants, the internal recycling of various nutrients has been studied using radiolabeled materials. Carbon (C) and sulfur (S) were shown to be re-translocated from leaves in a leaf age-dependent manner in cottonwood and *Populus tremula* × *P. alba*, respectively (Dickson, 1989; Hartmann *et al.*, 2000). These studies showed that expanding leaves daily re-translocate the remobilized nutrients mainly into source organs in the upper apical parts of the tree. On the other hand, old mature leaves re-translocated the nutrients mainly into lower parts of the tree (lower stem and roots). Dickson (1989) concluded that C transport from source leaves to sink tissues is controlled by both the vascular connections and relative sink demand.

Besides this daily re-translocation of nutrients, deciduous woody plants seasonally remobilize various nutrients including phosphorus from senescing leaves to perennial tissues during autumn, by rescuing these precious nutrients before the leaves

fall (Chapin & Kedrowski, 1983; Aerts, 1996; Keskitalo *et al.*, 2005; Netzer *et al.*, 2017b,a).

Recently we found that in *Populus alba*, P is remobilized from leaves during autumn senescence, and stored in perennial tissues such as twigs during winter as phytic acid (myo-inositol-1,2,3,4,5,6-hexakis phosphate: IP₆) (Kurita *et al.*, 2014, 2017). It is well-known that phytic acid is a major P-storage form in seeds (Raboy *et al.*, 2001). Kurita *et al.* (2014, 2017) showed that at the beginning of spring, IP₆ levels in twigs decreased, presumably to release P for new growth.

Although the seasonal distribution of P is becoming clearer, there is little information on how P is seasonally re-distributed in woody plants. For example, perennial tissues like twigs function as both sink and source. Twigs are not expected to be main sinks during the developmental season, although cambium zones must have sink ability for their secondary growth. However, during senescence, their sink ability increases as they begin to store nutrients for winter. In early spring, the twigs probably revert to become source tissues, and release the stored nutrients for new growing tissues.

In the stems of woody plants, cells form three-dimensional lattices and they transport water and solute along both the longitudinal axis and radial axes (Van Bel, 1990; Spicer, 2014). It is expected that the P re-translocation route also shifts dependent on the seasons. However, seasonal P re-translocation routes and mechanisms, and the contribution of phloem and xylem for it are currently unknown (Rennenberg & Herschbach, 2013).

It is quite difficult and very time-consuming to investigate the detailed mechanisms of nutrient re-translocation using trees grown outdoors in the field. Recently, we have developed a shortened annual cycle system to facilitate the investigation of the

seasonal phenology in the laboratory (Kurita *et al.*, 2014). This system consists of 3 stages. Stage 1 mimics spring/summer, stage 2 does autumn and stage 3 does winter, respectively (for detail, see method and Fig. S1c). After leaf fall in Stage 3, plants were transferred to stage 1 again and bud burst induced. In this system, poplar trees showed normal seasonal events (leaf coloration, leaf fall, bud burst) and seasonal P recycling events (e.g. the P remobilization from senescing leaves, the storing of IP₆ in dormant stems and the redistribution during budburst) (Kurita *et al.*, 2017).

In the present study, in order to clarify the pathways involved in phosphate re-translocation and its accumulation, we used this shortened annual cycle system in the laboratory with radio labeled phosphates. We succeeded in visualizing the spatiotemporally diverse P re-translocation using several autoradiography methods which cannot be applied to the field-grown plants.

Materials and Methods

Plant materials and culture conditions

Poplar shoots with 4-5 leaves were cut and rooted in 1/5 MS medium, and transferred into the pots containing vermiculite, that had been washed with tap water ten times and by distilled water five times. Potted cuttings were placed in tree culture chambers (LH-350S, NK system, Osaka, Japan) in turn from stage 1 to 3 under the conditions in “a shortened annual cycle system” as described before (Kurita *et al.*, 2014)(Fig. S1c). Briefly, stage 1 (25°C, 14h light) mimics spring/summer condition, and promotes vigorous plant growth. Stage 2 (15°C, 8h light) mimics autumn condition, and causes plants to experience short daylength and moderate cooler temperatures. Stage 3 (5°C, 8h light) mimics winter condition, and promotes leaf senescence. At this stage, plants cease apical growth. The leaves turn yellow and then fall.

Plants were fertilized with 1/5 MS medium (1L / 6 potted cuttings) once a week at stage 1. Afterwards they were cultured with distilled water only. In stages 1 and 2, plants at 3rd to 4th week, and in stage 3, plants at 3rd to 5th week, were used for the experiments.

Imaging using the real-time radioisotope imaging system

To add radiophosphorus, a "flap" was cut along a vein of 6th leaf in the manner shown in Fig.S1a (Biddulph & Markle, 1944), and 5 µl ³²P-phosphate (H₃³²PO₄) solution in 0.2M KH₂PO₄ (final concentration of 200 kBq µl⁻¹) was added. After 1 or 3 min, the flap was cut off because its strong signals disturb the imaging. Then, changes in ³²P distribution were observed using a real-time radioisotope imaging system (RRIS) established previously (Kanno *et al.*, 2012; Hirose *et al.*, 2013; Sugita *et al.*, 2013; Sugita *et al.*, 2016). The plant

was mounted on the fiber optic plate coated with 100 μm thickness of CsI (Tl) scintillator by vapor deposition (FOS, 100 mm $\times$ 100 mm $\times$ 5 mm, Hamamatsu Photonics Co., Hamamatsu, Japan). Two fiber optic plates were connected longitudinally. The beta ray emitted from ^{32}P in the sample interacts with the CsI (Tl) scintillator, and then is converted to visual light. Light was captured by the cooled CCD camera (AQUACOSMOS/VIMsystem, 640 $\times$ 480 pixel; Hamamatsu Photonics, Co, Hamamatsu City, Japan) with an image capture system (Aquacosmos/VIM system; resolution: 640 pixel $\times$ 480 pixel, Hamamatsu Photonics Co.) (Kanno *et al.*, 2012; Sugita *et al.*, 2013). The measurement was performed for three hours in a dark room at 20°C. The same measurements were repeated 3 times.

To analyze the velocity of ^{32}P transported in the vascular bundle, the signal intensity of the region of interest (ROI) was measured by ImageJ software. Two ROIs were set at the apex and the 6th leaf axil. Three ROIs were set at corners of FOS to measure background signals. Of the 3 replicates, the 2 trees which showed the highest signals were analyzed. The velocity was calculated from the distance between the apex and the 6th leaf axil and the difference between the times it took for the signal to reach the detection threshold. The detection threshold was defined as five times the standard deviation of the background signal intensity.

Whole plant autoradiography using imaging plate

^{32}P -phosphate (5 μl) in 0.2M KH_2PO_4 (final concentration of 200 Bq μl^{-1}) was added to 6th leaf as described above. Then, plants were cultured in each stage condition for 1 day. After 1 day culture, the 6th leaf was cut off because its strong signals disturb the imaging. The vermiculite was washed away from poplar roots with tap water. Plants were then

mounted on commercial xerographic paper, and wrapped with double layers of polyvinyl film. The sample was set on the imaging plate (Fujifilm, Tokyo, Japan) in the cassette case and exposed in a dark room for 3 days. The imaging plate was then measured using Typhoon FLA 9500 (GE Healthcare UK Ltd., Buckinghamshire, England). The same measurements were repeated 3 times.

Whole plant autoradiography using imaging plate with phloem girdling

Approximately 2 mm of bark from the upper or lower internodes of the 6th leaf of a plant at stage 1 was cut with a razor blade and peeled off, effectively disrupting phloem translocation in this region (Fig. S1b). Then immediately, 5 μ l ³²P-phosphate (KH₂³²PO₄) solution in 0.2 M KH₂PO₄ (final concentration of 1 kBq μ l⁻¹) was added to the 6th leaf as described above. Plants were cultured in stage 1 for 3 h or 1 day. After culture, the 6th leaf was cut off. Upper and lower internodes of 6th leaf were cut to prevent re-translocation of ³²P during contact. The vermiculite was washed away from poplar roots with tap water. Plants were then mounted on commercial xerographic paper and wrapped with double layers of polyvinyl film. The sample was set on the imaging plate in the cassette case and exposed in a dark room for 1 day. Then the imaging plate was measured using Typhoon FLA 9500. The same measurements were repeated 3 times.

Microautoradiography (MAR) of the stem

³³P-phosphate (5 μ l) in 0.2M KH₂PO₄ (final concentration of 37 or 74 kBq μ l⁻¹) was added to 6th leaf as described above. Then, plants were cultured in each stage condition for 1 day. ³²P is not suitable for obtaining high-resolution images because its energy is too high. Therefore, the experiments were performed by using ³³P which has a lower energy. ³³P

distribution in stems was detected by the MAR method for fresh-frozen sections, as described previously (Hirose *et al.*, 2014). Stem samples that were cut from a poplar in each stage, were frozen with liquid nitrogen. The frozen stems were embedded in a dedicated embedding medium (SCEM; SECTION-LAB Co. Ltd.) and sliced into 20 μm thick sections using a Cryostat (CM1850; Leica Microsystems, Wetzlar, Germany) set at -20°C . Sectioning was performed using the Cryo-Film transfer kit (SECTION-LAB Co. Ltd., Hiroshima, Japan) (Kawamoto, 2003; Hirose *et al.*, 2014).

Frozen sections were put on hydrophilic amino-coated glass slides (MAS-GP type A; Matsunami Glass Ind., Ltd., Osaka, Japan) coated with a photosensitive nuclear emulsion (Ilford Nuclear Emulsion Type K5; Harman Technology Ltd., Cheshire, UK) in the Cryostat, and contacted tightly at -80°C for approximately 1 month or more. A 1.2 μm thick polyphenylene sulfide (PPS) film was inserted between the frozen sample sections and the photosensitive nuclear emulsion on the glass slide to enable easy separation of the section and the emulsion after exposure. The results scarcely changed when the sample was exposed for more than 1 month.

After the exposure, the sections were removed from the glass slides in the cryostat set at -20°C in the darkroom. The glass slides were soaked in quarter-strength D-19 developer (Eastman Kodak Co., NY, USA) for 6 min at room temperature to develop the silver particles. Then, the slides were soaked in distilled water for 1 min to stop the development, followed by exposure to 0.83 M sodium thiosulfate for 4 min for fixation. Finally, the glass slides were soaked in distilled water for 10 min twice. The temperature of the darkroom was maintained at 20°C to ensure development.

In order to stain the sliced tissue, the sections were floated on distilled water for 1 min three times at room temperature to wash out the embedding material. Then the sections

were stained with 0.25 % toluidine blue for 30 sec and rinsed with water for 1 min. The sections were then mounted on a new glass slide using a glycerol-based mounting medium (SCMM; SECTION-LAB Co. Ltd.).

The stained sections and the silver particles developed by MAR were observed and photographed using a microscope (BX-60; Olympus, Tokyo, Japan). The tissue images and radiographs were then assembled using e-Tiling (Mitani Co., Tokyo, Japan) or ImageJ (<http://rsb.info.nih.gov/ij>) by its plugin, Grid/Collection stitching software (Preibisch *et al.*, 2009). The registration was performed on the tissue images and radiographs using ImageJ and LPixel's ImageJ Plugin, Lpx Registration software (<https://lpixel.net/products/lpixel-imagej-plugins/>) and GIMP software (<http://www.gimp.org>). The same measurements were repeated 3 times.

Autoradiography of the leaf axil and 3D construction

Fresh-frozen sections from the axil of the sixth leaf were prepared for autoradiography experiments using the imaging plate (BAS IP MS; GE Healthcare UK Ltd.). The thickness of the section was 20 μ m. The exposure time was about 76 h for samples in stage 1 at – 80°C. In stage 3, exposure time was about 22 h because the signal was stronger than that of samples in stage 1. The imaging plate was scanned with an FLA5000 image analyser (Fujifilm, Tokyo, Japan) at a resolution of 10 μ m.

After linear contrast enhancement and registration using ImageJ plugin StackReg (Thévenaz *et al.*, 1998), autoradiographs were converted to 3D images using ImageJ plugin Volume Viewer (<http://rsb.info.nih.gov/ij/plugins/volume-viewer.html>) and 3D Viewer (<http://imagej.nih.gov/ij/plugins/3d-viewer/>). The same measurements were repeated 3 times.

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244 **Whole plant autoradiography using imaging plate over dormancy period**

245 ^{32}P -phosphate (7 μl) in 0.2M KH_2PO_4 (final concentration of 700k Bq μl^{-1}) was added
 246 to 6th leaf of a plant at 4th week in stage 3 as described above. After leaf fall (at 8th week
 247 in stage 3) or after bud burst (at 2nd week in stage 1 of 2nd cycle), the vermiculite was
 248 washed away from poplar roots with tap water. The plant was then mounted on paper, and
 249 wrapped with a double layer of polyvinyl film. The sample was set on the imaging plate
 250 in the cassette case and exposed in a dark room for 15min or 1 day. Then, the imaging
 251 plate was measured using Typhoon FLA 9500. The same measurements were repeated 3
 252 times.

253

Results

Shift of longitudinal re-translocation routes of phosphorus between growing and senescence seasons.

To continuously observe how P is re-translocated from a leaf, we performed real-time radioisotope imaging using plants grown in the shortened annual cycle system. In stage 1, ^{32}P was re-translocated from the 6th leaf in both upward and downward directions, and gradually accumulated at apical parts (apex and young leaves) in 3h (Fig.1a, Video S1, Fig. S2). Within 30 min, ^{32}P was detected at the leaf axil of the applied leaves, and after 1 to 1.5 h, ^{32}P was detected at the shoot apex. Estimated transport velocities were about 0.015 mm s⁻¹ in tree A and 0.021 mm s⁻¹ in tree B (Fig. S2). On the other hand, in stage 3, ^{32}P was only re-translocated downwards from the 6th leaf. No signals in upper parts were detected within 3h (Fig.1b, Video S2). To observe ^{32}P transport and distribution in whole plants over longer periods, plants were cultured for 1 day after ^{32}P application, and contacted to imaging plates (Fig.2). In stage 1, ^{32}P was detected mainly in the upper parts, in agreement with the results of the real-time imaging experiment. During stages 2 and 3, ^{32}P was detected mainly in the lower parts (stem and roots), and slightly in the upper stem.

Longitudinal re-translocation of phosphorus via phloem.

To reveal which tissue (xylem and/or phloem) is involved in longitudinal re-translocation of P, changes in the re-translocation route of P were observed by phloem girdling using a tree in stage 1 (Fig. 3, S1b). The upper or lower phloem of a node attached to the 6th leaf was peeled off, and then ^{32}P was immediately applied to the 6th leaf. After 3 h or 1 day culture, the plant was set on imaging plate for ^{32}P detection. When the phloem was peeled off above the applied leaf, ^{32}P was detected only in the lower stem and roots

after 3 h (Fig.3a). After 1 day, ^{32}P was detected mainly in the lower stem and roots, and slightly in the upper stem and young leaves. When phloem was peeled off below the applied leaf, ^{32}P was detected only in the upper stem and young leaves after 3 h or 1 day culture (Fig.3b).

Visualization of P inflow from a leaf to the stem

To observe P inflow from a leaf to the stem in detail, serial autoradiographs of a leaf axil were converted to 3D images (Fig. 4a, b and S3). ^{33}P was used for more detailed observation in these experiments. In each stage, plants were cultured for 1 day after ^{33}P application. In stage 1, ^{33}P was detected throughout the stem, petiole and bud. In the stem, ^{33}P was mainly distributed in the phloem region on the side of applied leaf (Fig. 4a, S3a, Video S3). On the other hand, in stage 3, ^{33}P was strongly detected in the lower phloem on the applied side of the leaf and petiole (Fig. 4b, S3b, Video S4). ^{33}P was barely detected in the upper stem, axillary bud or the phloem opposite the petiole. At the junction between the petiole and the stem, there appeared to be formation of an abscission layer (Fig. 4b, S3b).

Shift of radial P re-translocation from a leaf between growing and senescence seasons.

To observe radial transport inside a stem during senescence, we used microautoradiography because of its higher resolution compared to conventional autoradiography using an imaging plate. Plants on which ^{33}P was applied to the 6th leaf were cultured for one day. ^{33}P localization in fresh frozen sections of the stem was detected. In the growing season at stage 1, ^{33}P was detected mainly in phloem and to a lesser extent throughout the section of the stem just below the 6th leaf (stem between 6th and 7th nodes) (Fig. 4c). As shown in the 3D reconstruction (Fig. 4a), the highest signals were recorded in

the phloem on the side of the ^{33}P applied leaf. This tendency was also observed in the upper stem (stem between 5th and 6th nodes) and the lower stem (stem between 9th and 10th nodes) (Fig. S4a, b).

During senescence at stage 3, ^{33}P was strongly detected in the phloem on the side of the applied leaf, and also in ray tissues and parenchyma cells around the pith (perimedullary cells) (Fig. 4d). ^{33}P was slightly detected both in the upper stem (stem between 5th and 6th nodes) and the lower stem (stem between 9th and 10th nodes) (Fig. S4c, d). Because ^{33}P accumulation was stronger in the stem between 6th and 7th nodes than in the stem between 9th and 10th nodes, the exchange from phloem to xylem probably occurred in the stem nearby the applied leaf rather than in the lower stem and roots.

Re-utilization of overwintering P stored in stem and roots by new growing tissues in spring.

To reveal how P stored in winter is re-utilized in spring, artificial overwinter experiments were performed in the shortened annual cycle system (Fig. 5). Plants, which had ^{32}P applied to the 6th leaf were cultured for about 1 month until leaf fall in stage 3. Just after leaf fall, ^{32}P was detected mainly in the lower stem and roots (Fig. 5a). Other trees were moved to stage 1 and bud burst induced. After bud burst in plants put in the second shortened annual cycle, ^{32}P remobilized from the 6th leaf was detected throughout the whole plant, particularly at apices and young leaves of new shoots (Fig. 5b).

Discussion

Experimental system

Seasonal changes in re-translocation of phosphorus (P) have been investigated by measurement of phosphate contents in organs (Chapin & Kedrowski, 1983; Keskitalo *et al.*, 2005; Kurita *et al.*, 2014, 2017; Netzer *et al.*, 2017a,b).

In this study, we have attempted to realize how such P is re-translocated between organs. Our shortened annual cycle system succeeded in reproducing the annual phenology of poplar trees in a laboratory (Kurita *et al.*, 2014, 2017), and here we have used this system to visualize the seasonal changes of P re-translocation in poplar trees using radiolabeled phosphate at both whole plant level and tissue level (Fig. 6).

Seasonal shifts of P re-translocation routes from a leaf.

At stage 1, P was re-translocated from the 6th leaf mainly to younger leaves and the shoot apex (Fig. 1a, 2). These results correspond with previous studies on re-translocation of C and S from leaves (Dickson, 1989; Hartmann *et al.*, 2000). On the other hand, in stage 3, although the applied leaf position (6th) was the same, P was mainly re-translocated to the lower stem (Fig. 1b, 2). This seasonal difference was more obvious in 3D images (Fig. 4). The upward transport clearly ceased at stage 3 compared with stage 1. P re-translocation to the axillary bud also changed between stage 1 and stage 3 (Fig. 4a, b). These results indicated that seasonal shifts of sink tissues and of longitudinal re-translocation routes occur between the growing season (stage 1) and senescence (stage 3). In stage 1, the apical meristem is active and trees keep growing. Then, during stages 2 to 3, trees cease apical growth and leaves start to senesce. Loss of sink ability in the apex,

younger leaves and axillary buds may lead to the seasonal transition of re-translocation routes. In the northern red oak, it was observed that the C allocation pattern changed with the timing of flushing of new leaves (Dickson, 1989).

Real-time observation of P re-translocation from a leaf

Real-time imaging revealed a rapid flow of phosphate in poplar plants (Fig. 1). In stage 1, ^{32}P was re-translocated from the 6th leaf both downward and upward, and ^{32}P signals were detected at the apex within 1 to 2 h (Fig. 1a, S2). This rapid P re-translocation probably occurred via phloem because early P re-translocation was suppressed by phloem girdling (Fig. 3). The mechanism of phloem transport is generally explained by the Münch Theory as mass flow driven by an osmotically generated pressure gradient (Münch, 1930; De Schepper *et al.*, 2013). Windt *et al.* (2006) measured velocities of the downward phloem flow in poplar using NMR imaging, and recorded a velocity of $0.34 \pm 0.03 \text{ mm s}^{-1}$ in the daytime (Windt *et al.*, 2006). Schepper *et al.* (2013) found that phloem speed in angiosperms is roughly within the same range, around 0.17 mm s^{-1} (De Schepper *et al.*, 2013). By the detection of ^{32}P signals, we estimated that the transport velocity was about 0.015 mm s^{-1} to 0.021 mm s^{-1} (Fig. S2). It should be noted that this value, which is considerably slower than previously reported, may probably show under-estimation. This result was partly expected because the thickness of poplar stems and leaves prevent instant detection of ^{32}P in the present system. In poplar stems, ^{32}P was localized in internal tissues (primarily the phloem), therefore its detection might be hindered in inverse proportion to the thickness of the outer tissues. It is believed that this decrease in signal intensity caused the signal to fall below the detection limit, leading to detection

delays. Therefore, at this time, we can only discuss the direction of P transport and the tissues that play a role of P re-translocation.

Phloem-xylem exchange of P in stem.

In the growing season, P re-translocated from leaves was exchanged from phloem to xylem in the stem, and transported to apical parts via xylem over longer periods (Fig. 3). Microautoradiography also showed the presence of re-translocated ³³P in both phloem and xylem (Fig. 4c). Such circulation using both phloem and xylem to transport nutrients coincides with previous reports in herbaceous plants (Biddulph *et al.*, 1958b) or reports about other nutrients in woody plants (Hartmann *et al.*, 2000; Herschbach *et al.*, 2012). However, it is unclear how P is re-translocated from phloem to xylem. Pfautsch *et al.* (2015) demonstrated the radial flow of water from phloem into xylem in *Eucalyptus saligna* by injection of a fluorescent dye. The radial transfer of water occurs predominantly via the symplast of ray parenchyma cells. Radial P transport may also occur symplastically along with the water flow. Although P re-translocation occurred predominantly via phloem, phloem-xylem exchange would be an advantage especially for long-distance transport in a tall tree. Because the xylem flow velocity ($1.60 \pm 0.09 \text{ mm s}^{-1}$) is much faster than the phloem flow (Windt *et al.*, 2006), remobilized nutrients can be transported to upper parts in a shorter period.

P re-translocation and winter storage in stem.

During the growth season, poplar trees re-translocate P from source tissues such as mature leaves to the growing sink tissues via phloem and xylem. The stem cambium may

also have sink ability because the stem diameter increased at stage 1 (Baba *et al.*, 2018), and ^{33}P was detected in the entire section including the cambium region (Fig. 4c).

On the other hand, during senescence, stem and roots function as sink tissues for winter P storage (Fig. 2, 5a). In stage 3, re-translocated ^{33}P was more strongly detected in xylem parenchyma cells of the stem just below the 6th leaf (stem between 6th and 7th nodes) than in other parts (Fig. 4d, S4c, d). Thus, the remobilized P from a leaf is thought to be immediately transferred from phloem to xylem, and stored in ray cells and perimedullary cells (Fig. 6). Phloem parenchyma cells also play a role in winter storage. We have previously reported that winter P is stored as IP_6 in the globoid-like structures in protein storage vacuoles at phloem and xylem parenchyma cells in poplar twigs (Kurita *et al.*, 2017). ^{33}P localization using microautoradiography (Fig. 4d) agreed with the localization of globoid-like structures. Netzer *et al.* (2017a) also reported P accumulation in the cambial zone and wood ray parenchyma cells of *Populus* $\times$ *canescens* during dormancy.

Re-allocation of P from the stem to new shoots in spring

After bud burst, stored P was re-allocated to the whole plant, and strongly detected in young leaves and the shoot apex (Fig. 5). This indicates that poplar stem and roots are seasonally reversible sinks, that function as a source of P during early spring. We succeeded in demonstrating that P remobilized from leaves is recycled beyond winter for spring growth in *Populus alba*. Interestingly, we supplied ^{32}P to only a single 6th leaf before winter, but in spring, ^{32}P was re-allocated to the whole plant from the stored parts. This means that P which is stored from a leaf in winter, is re-allocated to the whole plant and mixed with P from other source tissues in spring.

Conclusion

Seasonal P recycling is thought to be an adaptive strategy tailored to the lifestyle of deciduous plants. In this paper, we visualized how P is seasonally re-translocated in juvenile poplar trees, and clearly showed that re-translocated P from leaves was reused for spring growth after overwintering.

The next step is to try to discover how deciduous trees regulate such seasonal P re-translocation depending on environmental cues and/or internal P status. The seasonal cycle of growth and dormancy, and the molecular mechanisms by which day length and temperature signals regulate the annual cycle have been well studied in *Populus* (Maurya & Bhalerao, 2017). Identification of phosphate transporters and expression profiles according to tissues and P availabilities have also been reported (Loth-Pereda *et al.*, 2011; Kavka & Polle, 2016). In herbaceous plants, local and systemic signaling for Pi starvation is becoming increasingly understood (Chien *et al.*, 2018).

Although investigations of seasonal phenomena in trees take longer, the shortened annual cycle system enables us to accelerate it, and to perform seasonal experiments using transgenic trees more easily. The combination of laboratory and field studies will be important in the elucidation of this re-translocation mechanism in mature tall trees in the field, and will contribute to our understanding of the annual nutritional balance of the entire tree.

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448 **Author Contribution**

449 YK and TM designed the study and wrote the manuscript. SK and RS instructed
 450 experiments using RRIS, and YK performed it with SK and RS. AH instructed
 451 experiments using MAR, and YK performed it with AH. KT and TMN advised
 452 experimental design using RRIS and MAR. KB instructed on dendrology. MO, HF and
 453 KI supported YK's experiments in Kobe university. All authors discussed the results and
 454 advised on the manuscript.

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Figures

Fig. 1 Real-time imaging of ^{32}P in *Populus alba*. (a) At stage 1. (b) At stage 3. Pink color triangles indicate the position of 6th leaf. The 6th leaf was shielded by acrylic board because its strong signals disturb measurement. bar = 2 cm.

Fig. 2 Whole plant autoradiographs of *Populus alba* trees in a shortened annual cycle system. Images after one day of ^{32}P application at each stage. Pink color triangles indicate the position of 6th leaf. The 6th leaf was cut off because its strong signals disturb measurement. In stage 2 and 3, the stem was cut because the plant was too large for the imaging plate. bar = 4 cm.

Fig. 3 Whole plant autoradiographs of *Populus alba* trees with phloem girdling. **(a)**

Plants peeled of their upper bark from the 6th leaf were cultured for 3 h or 24 h. **(b)** Plants peeled of their lower bark from the 6th leaf were cultured for 3 h or 24 h. Plants at stage 1 were used for experiments. ³²P solution was added to the 6th leaf indicated by pink color triangles. Blue color triangles indicate the position of phloem girdling. After cultivation, upper and lower internodes of the 6th leaf were cut to prevent further re-translocation of ³²P during contact. bar = 4 cm.

Fig. 4 Side view of the 6th axil and microautoradiographs of the stem between the 6th and 7th nodes of *Populus alba*. (a) Side view of the 6th axil at stage 1. ³³P autoradiograph sections were assembled into 3D images using ImageJ. (b) Side view of the 6th axil at stage 3. The white color triangle indicates abscission layer during formation. (c) The stem between 6th and 7th nodes in stage 1. Left: section stained by toluidine blue. Middle: microautoradiograph of the section. Right: merged image. Green color triangles indicate the position of the 6th leaf. (d) The stem between 6th and 7th nodes in stage 3. Bar = 0.5 mm.

Fig. 5 Whole plant autoradiographs of *Populus alba* trees in the shortened annual cycle system. Poplar trees in which ^{32}P was applied to the 6th leaf at 4th week in stage 3 were subsequently cultured in stage 3. (a) Image after 4 weeks of ^{32}P application (just after leaf fall). (b) Image after 6 weeks of ^{32}P application. After 4 weeks cultivation, plants were moved to stage 1, and bud burst was induced. Pink color triangles indicate the position of the 6th leaf. The stem was cut because the plant was too large for the imaging plate. bar = 4 cm.

Fig. 6 Overview of the seasonal change of P re-translocation in *Populus alba*. In the growing season, P is mainly re-translocated from the 6th leaf to upper tissues (young leaves, apex) via phloem. P is re-translocated also to the axillary bud. During senescence, P is re-translocated from the 6th leaf to lower tissues (stem, roots) via phloem. P is exchanged from phloem to xylem near the 6th leaf, and accumulated in ray cells and perimedullary cells for winter storage.

Figure legends of Supporting Information

Fig. S1 Surgical treatments on *Populus alba* trees and a summary diagram of culture conditions.

Fig. S2 Transition of ^{32}P signal intensity (intensity cm^{-2}) of the apex and the 6th leaf axil.

Fig. S3 ^{33}P autoradiographs of leaf axil of 6th leaf in stage 1 using imaging plate.

Fig. S4 Microautoradiographs of the stem between 5th and 6th nodes.

Video S1 Real-time imaging movie of ^{32}P in *Populus alba* at stage 1.

Video S2 Real-time imaging movie of ^{32}P in *Populus alba* at stage 3.

Video S3 3D image of the 6th axil of *Populus alba* at stage 1.

Video S4 3D image of the 6th axil of *Populus alba* at stage 3.