

Dormancy-induced temporal up-regulation of root activity in calcium translocation to shoot in *Populus maximowiczii*

Jun Furukawa, Masafumi Kanazawa and Shinobu Satoh

Graduate School of Life and Environmental Sciences, University of Tsukuba, Tennodai 1-1-1, Tsukuba 305-8572, Japan

Corresponding author: S. Satoh, E. mail: satoh.shinobu.ga@u.tsukuba.ac.jp, Phone: +81-29-853-4672, Fax: +81-29-853-4579

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Abstract: To explore seasonality of root functions, we analyzed the concentrations of 8 minerals in leaves of *Populus maximowiczii* (Japanese native poplar) by inductively coupled plasma atomic emission spectroscopy. These concentrations were used as indices of root mineral translocation activity. In leaves close to the shoot apex, dramatic increases in Ca concentration, and similar but slight increase in Mg and Mn, were observed after the onset of dormancy. Because of the constant concentration of Na, which is not essential for plant growth, the increase of Ca concentration was mainly derived from not by the increase of transpiration rate but by the enhancement of root activity of xylem loading. Leaf Ca concentration in August 2010 was approximately fivefold higher than before dormant bud formation. To investigate the shifts in Ca-translocation activity during dormancy induction, we grew saplings hydroponically under light- and temperature-controlled conditions and subsequently analyzed the distribution of ^{45}Ca absorbed by roots using a Bio-Image Analyzer. In this pulse chase experiment, the enhancement of ^{45}Ca translocation to the shoot was not observed in early dormancy. This suggested the increase of leaf Ca in early dormancy was caused by the Ca loading into root xylem vessels using the root Ca absorbed before the onset of dormancy. These changes in mineral translocation activities indicate that alterations in Ca distribution are most probably triggered by bud dormancy. Furthermore, several root functions were regulated by the dormancy induction process.

Keywords: calcium, dormancy, mineral, *Populus maximowiczii*, translocation

Abbreviations: ABA, abscisic acid; ICP-AES, inductively coupled-plasma atomic emission spectrometry

Introduction

The seasonal cycle of growth and dormancy is distinctly characteristic of perennial plants, and the phase shift from growth to dormancy is a basic adaptation of trees to their environment (Jansson et al. 2010). The transition of meristems into and out of dormant bud is crucial for plant growth and survival. These transitions are linked to bud flush and bud set, restricting the growing season. Woody plants use environmental cues, such as photoperiod and temperature (Welling et al. 2002), to transition between maximum growth and timely dormancy-induced meristem protection against hazardous frosts. The initiation of cold acclimation under reduced day length increases endogenous abscisic acid (ABA) levels and reduces endogenous gibberellic acid (GA) levels (Olsen et al. 1997, Welling et al. 1997, Møllmann et al. 2005).

Although physiological and molecular changes have been thoroughly investigated during dormancy induction and break in shoots of various woody plants (Arora et al. 2003, Ruttink et al. 2007), the effects of the dormancy on the root functions and activities have not been well studied yet. Moreover, although woody plants use environmental cues, such as photoperiod and temperature (Welling et al. 2002), as signals to trigger transitions between rapid growth and dormancy in shoot, the trigger for the shift of root function induced by the dormancy has been unclear possibly because of no light perception in root and relatively constant ground temperature. Based on our comprehensive analysis of xylem sap components (Furukawa et al. 2011), increases in several components, including K, Ca and Mg, in

winter season were observed. If the dormancy-induced changes in the root functions are identified, the phenomenon will be a promising model for investigating the phase shift of root activity under dormancy. In this research, we focused our attention on root activity affected by the dormancy and effects of early stage dormancy on mineral accumulation in leaves. Because translocation of minerals to the shoot depends mostly on root functions that uptake minerals from rhizosphere and supply dissolved ions to the xylem vessels, the effect of dormancy on shoot mineral profiles reflects dormancy-regulating root functions.

The lack of a woody plant model in molecular explorations has certainly hampered progress in the study of tree seasonality. However, the entire *Populus trichocarpa* genome was recently sequenced (Tuskan et al. 2006) and expressed sequence tags of *Populus* were identified (Kohler et al. 2003, Sterky et al. 2004). These advances have attracted molecular biological research on deciduous trees. Poplars initiate dormancy primarily in response to short day lengths (Sylvén 1940, Nitsch 1957), and increased genetic and physiological understanding of dormancy initiation and break makes poplar a highly suitable deciduous tree model for investigating growth rhythms. To utilize these advantages, we also employed poplar as our experimental plants for investigating the shift of root function.

In this study, we analyzed seasonal changes in leaf mineral concentrations from 2009 to 2010 to identify the rhythms of root function related to dormancy and Ca uptake from the root and an allocation between root and shoot was investigated using radioisotope, ^{45}Ca and autoradiography.

Materials and methods

Plant material and climate

Leaf samples were harvested from *Populus maximowiczii* growing in Tsukuba City, Ibaraki, Japan (36°05'N, 140°07'E; 25 m elevation). *P. maximowiczii* is a poplar native to Japan, it is in the same phylogenetic clade as *P. trichocarpa* whose whole genome has been sequenced (Tuskan et al. 2006). The original shoot cuttings had been obtained 2001 from the lateral branches of a mature *P. maximowiczii* planted in the National Science Museum, Tsukuba Botanical Garden; the ID number of the original plant was TBG10713, and used for the propagation with cutting culture. Two trees grown in pots with commercial culture soil for 5 years after cutting culture were used for this study. Thus, all plants used had the same genetic background. Since the plants prepared for the experiments were mini-

mum, to avoid the pseudo-replication problem (Hurlbert 1984), at least two independent experiments were performed and the only consistent results were focused.

As for meteorological data at the experimental location, day length data in 2009 and 2010 was obtained from National Astronomical Observatory of Japan (<http://www.nao.ac.jp/E/index.html>) and air temperature data in both years were from Japan Meteorological Agency (<http://www.jma.go.jp/jma/indexe.html>) (Fig. 1).

Collection of leaf samples

Two potted plants (Plant A and B) were grown outdoors in 15 L of soil augmented with organic fertilizer (fermented oil cake) in March 2009 and 2010. Leaf samples were collected on August 23, September 19, and October 21, 2009. Leaf abscissions started at a lower node position at the end of September. By November, all leaves had been shed. On February 20 2010, we pruned the main and lateral branches of Plant A and, on March 13 of the same year, we pruned Plant B in the same manner, to get cuttings for another propagation. After pruning the main and lateral branches in 2010, we continued collecting leaves from the same plants on April 19, May 25, June 16, July 18, August 19, September 17, and October 19, 2010. In 2009, the 3rd, 8th, and 17th expanded leaves behind the main branch shoot apex were harvested, and in 2010, we collected the first expanded leaves behind the apices of the main and lateral branches.

Determination of metal concentration

Harvested leaves were rinsed with Milli-Q (Millipore, Billerica, MA) solution and dried at 70°C for 2 days. After drying, whole leaves were homogenized with a mortar and pestle, and the samples (50 mg each) were pre-digested overnight in a solution of 40% nitric acid and 10% hydrogen peroxide. Subsequently, we digested samples in concentrated nitric acid at 140°C. To measure metal concentrations, we first filtered digest solutions diluted with Milli-Q through 0.45- μm membrane filters (Millipore), after dilution with 0.1 N HNO_3 , we determined the concentrations of sodium (Na), magnesium (Mg), phosphorus (P), potassium (K), calcium (Ca), manganese (Mn), iron (Fe) and nickel (Ni) by inductively coupled plasma-atomic emission spectroscopy (ICP-AES) (Optima 7300 DV; PerkinElmer, Waltham, MA) at the Chemical Analysis Center, University of Tsukuba. To calculate the concentrations of these elements, we obtained standard solutions from Wako Pure Chemical Industries (Osaka, Japan).

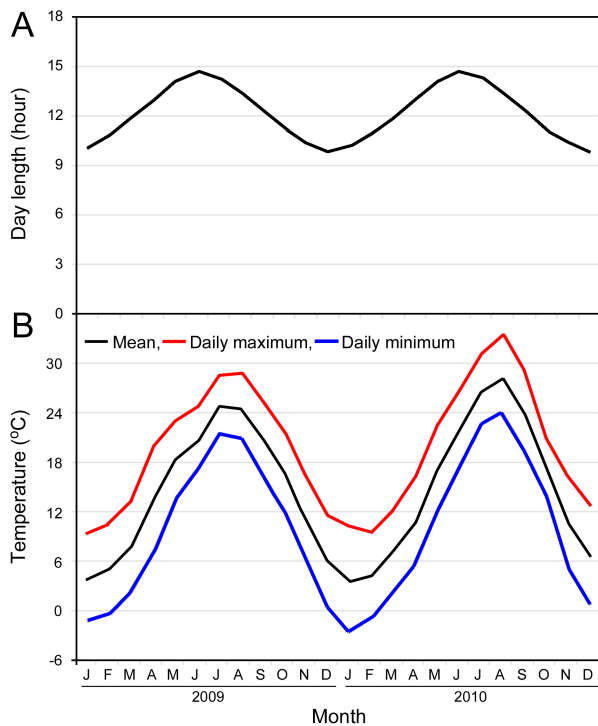


Fig. 1. Day length (A) and air temperature (B) at the experimental location in 2009 and 2010. The data were obtained from National Astronomical Observatory of Japan (<http://www.nao.ac.jp/E/index.html>) and Japan Meteorological Agency (<http://www.jma.go.jp/jma/indexe.html>).

Assay of ^{45}Ca uptake from the root

The plants used for the ^{45}Ca assay were also prepared by cutting propagation. The plants grown under long-day (LD) condition (16 h light, 8 h darkness; fluorescent light, 26°C, according to Jeknić and Chen 1999) in the light- and temperature-controlled room at least 6 months were used as mother plants and rhizogenesis was induced in cutting branches grown in culture soil under LD condition for 1 month. Subsequently, plants were transferred to hydroponic culture in a HYPONeX (HYPONeX Japan, Osaka, Japan) solution (the HYPONeX:tap water ratio was 1:1000 (w/w)) for an additional month. Plants grown under LD conditions for 2 months were subsequently subjected to a range of photoperiod conditions: LD conditions for 2 additional months, short-day (SD) conditions (8 h light, 16 h darkness; fluorescent light, 26°C, according to Jeknić and Chen 1999) for 3 weeks, and SD conditions for 3 months. Immediately afterward, plants were transferred to 300 mL of 1/8 strength Hoagland's solution containing $^{45}\text{CaCl}_2$ (0.9-1.8 MBq; PerkinElmer) for 5 days under the respective treatment conditions. ^{45}Ca radioactivity in

the plants was detected with a Bio-Imaging Analyzer BAS 1800-II (FujiFilm, Tokyo, Japan), the radioactivity in each image was normalized against two reference spots (containing 1 and 10 kBq ^{45}Ca) exposed with the samples. The pixel value was obtained with ImageJ software (Abramoff et al. 2004). The total amount of ^{45}Ca radioactivity absorbed in whole plant was obtained and the radioactivities of shoot and root tissues were calculated as a percentage.

Results

Growth of P. maximowiczii under natural conditions

By June 2009, the two potted plants (Plant A and B) were approximately 2 m tall. By the end of August 2009, growth ceased when plants were about 4 m tall and dormant buds formed. The plants were approximately 1 m tall on April 2010. Leaf expansion and elongation of main and lateral branches began early in May. Formation of lateral branch dormant buds occurred at the end of May and the growth of lateral branches stopped. On the main branches, dormant buds formed at the end of June and growth ceased when plants were about 2 m tall. Leaf abscissions started at a lower node position in August and by November, all leaves had been shed.

Accumulation profiles of mineral elements in leaves of P. maximowiczii

In 2009, we measured the concentrations of six elements in the 3rd, 8th, and 17th expanded leaves behind the shoot apex of the main branch (Fig. 2). Concentrations of Mg, Ca, and Mn had increased in leaves in the 3rd position behind the apex by September following the induction of dormancy. Magnesium concentration in leaves in the 3rd position behind the apex increased about 1.6-fold from August to September. This trend stopped between September and October. Mg concentration was constant in leaves in the 8th position. No discernable pattern in Mg concentration was observed in leaves in the 17th position. In October, Ca and Mn concentrations in leaves in the 3rd position were approximately 1.6-fold higher than concentrations in August. No obvious temporal trends in Ca and Mn concentrations were detected in leaves in the 17th position. K and Fe concentrations in leaves in the 3rd position were constant over time, but in leaves in the 17th position, increases occurred in September. Zn concentrations in leaves in the 3rd position decreased slightly as the seasons progressed.

Based on observed accumulations of Mg, Ca, and Mn in higher node leaves over time in 2009, we

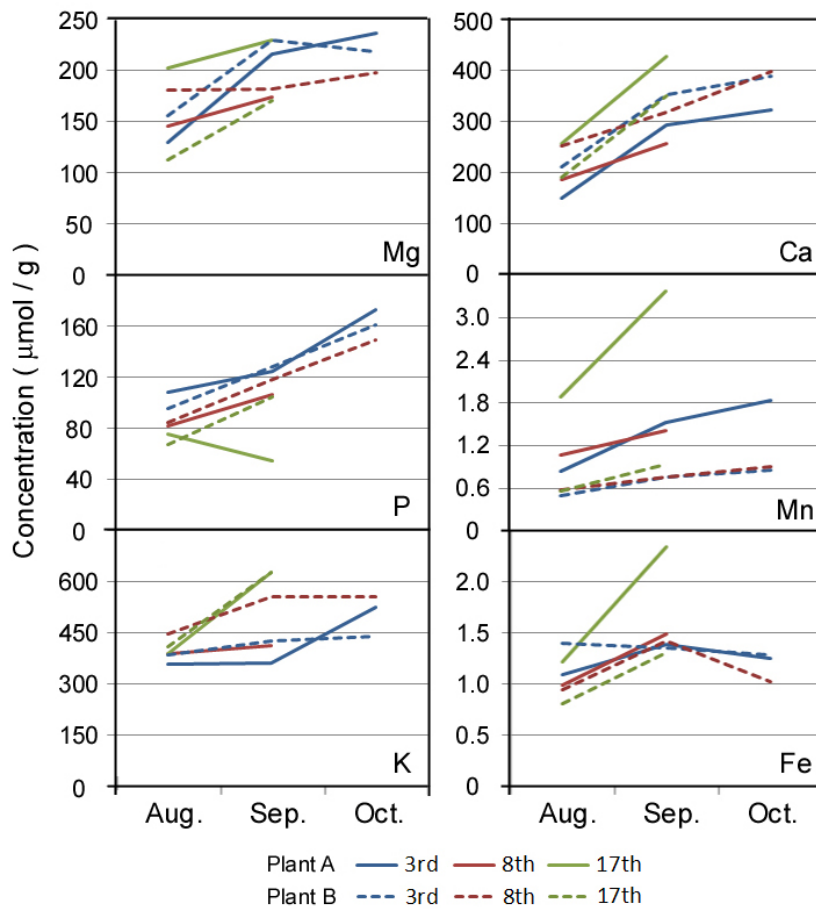


Fig. 2. Elemental profiles of Mg, P, K, Ca, Mn and Fe in leaves in the 3rd, 8th, and 17th positions behind the shoot apex collected in each sampling month of 2009 from two potted *Populus maximowiczii* plants. Dormant buds formed at the end of August. The data not indicated means “leaf shed and no data obtained.”

proceeded to measure concentrations of 8 elements in the first expanded leaves of the main and lateral branches in 2010. In contrast to seasonal increases observed in 2009, several accumulation patterns were observed in 2010 (Fig. 2 and 3). Mg increased monotonically from April to August in the main and lateral branches and decreased slightly in September immediately preceding leaf abscission (Fig. 3). Increases in Mn concentration began in July and June in the main and lateral branches, respectively. This 1-month difference between branch orders was synchronized with the dormant bud set on each branch type. In 2010, the formation of dormant buds occurred between June and July on the main branches and between May and June on the lateral branches. Concentrations of Ca gradually decreased from April to June, but increased dramatically in July (Fig. 3). Ca concentration increased until August, reaching a level approximately fivefold higher than in June. Similar increases in Ca also occurred in the lateral branches, although Ca accumulation in these branches began in June. This phase difference was also synchronized with the dormant bud set on each branch order. Mn concentrations tracked temporal

changes closely similar to those of Ca. In the main branches, Mn gradually decreased between April and June, then increased from July to August. In contrast to temporal patterns in leaves in the 3rd, 8th, and 17th positions in 2009, decreases in K concentration occurred in the main and lateral branches from June onward in 2010 (Fig. 3). The onset of this decrease in K concentration was not fully synchronized with the dormant bud set on either branch order.

Calcium uptake and translocation regulated by environmental factors

We measured ^{45}Ca uptake in plants subjected to different photoperiods for different lengths of time. Control measurements were made on plants following a 5-day application of ^{45}Ca to the roots after LD treatment over 4 months. ^{45}Ca accumulated to high concentrations in leaf midribs, stems and roots (Fig. 4A). The isotope also accumulated in leaf blades around the shoot apex. ^{45}Ca accumulation was obviously higher in leaves around the apex than in those located in lower node positions. In the plants treated under SD conditions for 3 weeks, ^{45}Ca

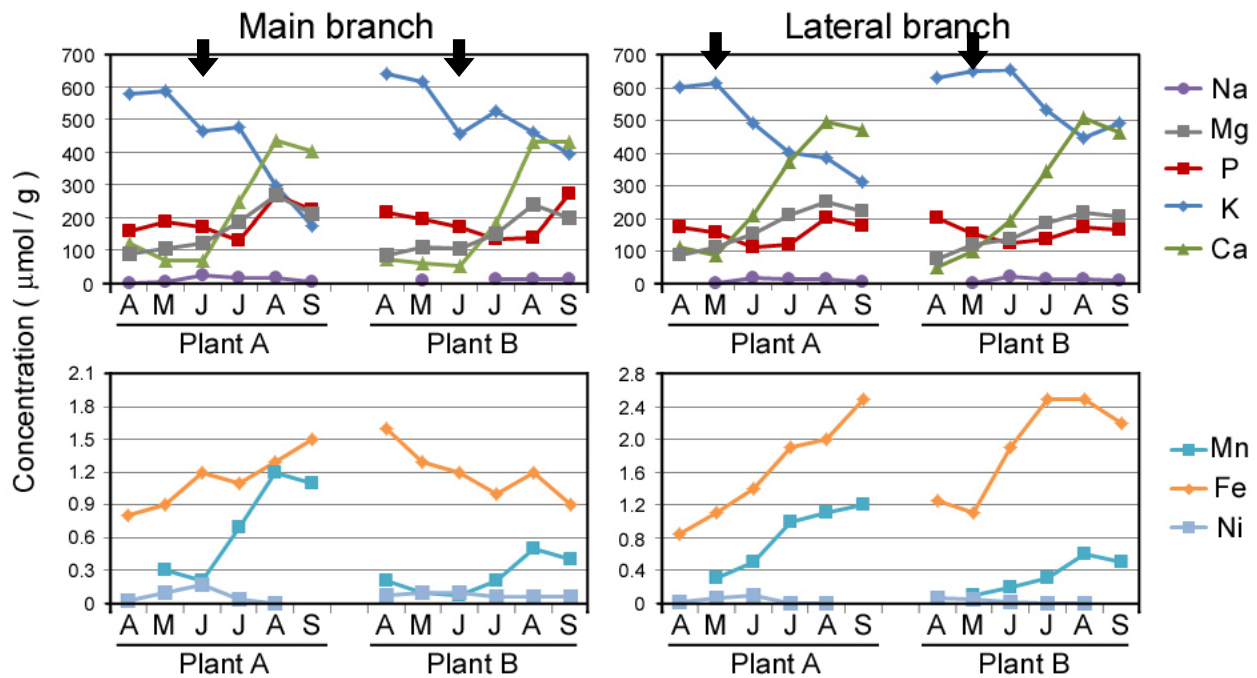


Fig. 3. Elemental profiles of Na, Mg, P, K, Ca, Mn, Fe and Ni in the first leaves behind the shoot apex collected from the main and lateral branches of two potted *Populus maximowiczii* plants from April to September in 2010. Arrows indicate the timing of dormant bud formation on each branch. The data not indicated means “below the detection limit.”

accumulation in the shoot was similar to that in LD-treated plants (Fig. 4B), however, lateral veins in fully expanded leaves also contained abundant ^{45}Ca (Fig. 4B, arrows). Ca allocation between the shoot and root differed between LD-treated plants and those treated under the SD condition for 3 weeks; the signal density of ^{45}Ca in root tissue was lower in the latter treatment (Fig. 4A and B). Plants treated under SD conditions over 3 months had elevated ^{45}Ca accumulation in the roots (Fig. 4D). Under the 3-month SD conditions, isotope accumulated in leaf midribs, but the signals from stems and lateral vein were weaker than those in plants grown under LD or 3-week SD conditions (Fig. 4A, B and C). Across all treatments, ^{45}Ca accumulation was minimal in shoot apices and dormant buds.

Discussion

Growth of P. maximowiczii under natural conditions

Woody plant meristems have to change appropriately into dormant bud for balancing between maximum growth and the protection against hazardous frosts. However, in 2010, dormant bud formation was initiated in late May and June on the lateral and main branches, respectively. This transition was unusual and about 2 months earlier than an average year and it occurred under LD conditions. At the experimental

location, day lengths on May 21 and June 20 were about 14.0 h and 14.5 h, respectively (Fig. 1A). The trigger for this early formation of dormant buds was not obvious, but occasional frosts in early spring may have been a factor. Regardless of the details, our observations indicate definitively that changes in mineral concentrations in leaves reflecting mineral translocation activity in root were not induced by SD conditions but rather by the onset of dormancy.

Mineral accumulation profiles in leaves

Among the minerals investigated, a distinct pattern in Ca concentration was observed in both years, especially in leaves around the shoot apex. Ca concentrations in 2010 dramatically increased after dormant bud formation in both the main and lateral branches (Fig. 3). In 2009, similar increases in Ca concentration occurred in leaves in the 3rd and 8th positions following dormant bud formation at the end of August (Fig. 2). Calcium taken up by the roots is transported acropetally almost exclusively via the transpiration stream (Marschner 1995). Different from other minerals, Ca is mainly deposited in the apoplast and binds to various cell wall sites, particularly the carboxyl groups of pectins. Once Ca is bound to the cell wall compartments, it is largely unavailable to further processes in plant metabolism (Lautner and Fromm 2010). This reduced mobility of

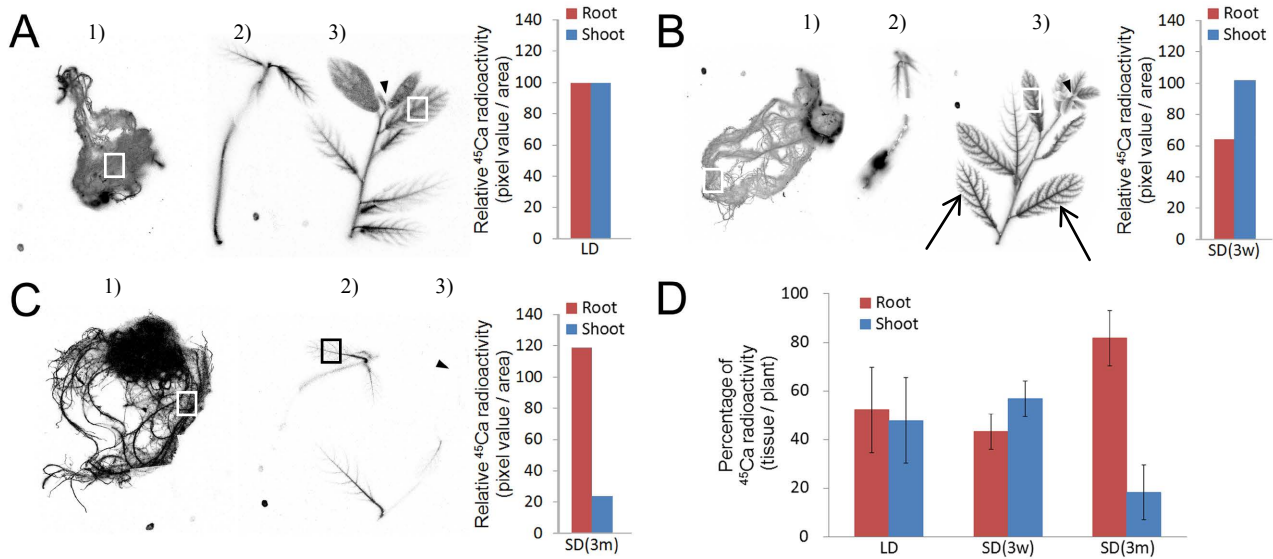


Fig. 4. Autoradiograms showing the distribution of ⁴⁵Ca radioactivity 5 days after isotope application to the root of *Populus maximowiczii*. Plants were grown hydroponically for 1 month under long-day conditions and then subjected to long-day conditions for 2 additional months (A; LD), short-day conditions for 3 weeks (B; SD(3w)), and short-day conditions for 3 months (C; SD(3m)). The image obtained were 1) root, 2) lower stem and 3) upper stem in each treatment. Radioactivity of each image was normalized by reference spots exposed with the samples. White or black rectangles indicate the root and leaf positions at which relative ⁴⁵Ca radioactivity in each treatment was determined. Arrowheads indicate the shoot apices of each plant. In the 3-weeks SD treatment, lateral veins in fully expanded leaves contained abundant ⁴⁵Ca (B, arrows). The percentages of ⁴⁵Ca radioactivity in whole shoot and root tissues are indicated with standard deviation (D).

deposited Ca indicates that Ca concentration increases with leaf age. However, Ca concentrations in leaves in the 3rd, 8th, and 17th positions in 2009 did not show an age-dependent order, such as 3rd < 8th < 17th (Fig. 2). Therefore, dormancy-induced Ca accumulation is attributable to changed Ca translocation activity from root to shoot. Several older and more recent studies indicate that the Ca concentration increases in tree leaves are dependent on leaf age. In yellow poplar (*Liriodendron tulipifera*), the Ca concentration rapidly increases from 1.38% in June to 2.3% in mid-August (Smalley 1976), and the rise in Ca concentration is more prominent in deciduous trees than in evergreen species (Ralhan and Singh 1987). However, these Ca increases are leaf age-dependent and no dormancy-induced pattern was observed. The Ca content of the apical meristem is reported to be significantly higher than that of potassium and phosphorus (Lautner and Fromm 2010). Ca accumulation in leaves around the shoot apex might be an accompanying effect of Ca accumulation in the shoot apical meristem because leaves are shed in the winter season. The Ca accumulation observed here suggests that Ca uptake and/or translocation activity was enhanced at the initial stage of dormancy and these dormancy-induced shift of root function was not identified so far.

In our experiment, magnesium and manganese gradually accumulated in leaves around the shoot

apex in both 2009 and 2010 (Fig. 2 and 3). Different from Ca, slight dormancy-induced accumulation was observed. K concentrations in 2009 were almost unchanged through time in leaves around the shoot apex (Fig. 2), however, in 2010, K gradually decreased from spring to autumn (Fig. 3).

Once dormancy has set in, Mg, P, K, and Ca are re-translocated from the leaf blade and transferred mainly to the stem especially the cortex and pith tissues, although apoplast-deposited Ca is less mobile and not so readily re-translocated (Eschrich et al. 1988). In our measurements, two patterns of mineral concentration decrease were observed. The first was of short duration and occurred immediately before leaf fall; Mg and Ca followed this pattern. The second was a long decrease over several months, as observed for K (Fig. 3). In comparison to Mg and Ca, K is a more easily leached element (Park and Cho 2003). Hence decreasing K concentration in our experiment resulted from the re-translocation activity of plant tissues and from leaching in the rainy season from mid-June to mid-July 2010.

In outdoor grown poplar, the concentration of Na, which is a non-essential element for plant growth and translocated passively on the transpiration stream, was not changed after the onset of dormancy (Fig. 3). This might suggest the transpiration-dependent mineral accumulation was not affected by the onset of dormancy.

Changes in Ca uptake and translocation activity in root induced by dormancy

Increased Ca concentration in leaves suggests that changes in Ca uptake and/or translocation in root were induced by dormancy. We analyzed ^{45}Ca distribution with pulse chase experiment in the SD treated plants to explore Ca uptake and translocation in more detail. ^{45}Ca translocation to the shoot gradually decreased (Fig. 4A-C). The ^{45}Ca distribution assay results largely reflected the movement of ^{45}Ca absorbed by the root, therefore, re-translocation of Ca from shoot tissues under SD conditions and the root Ca absorbed before the root ^{45}Ca application was barely discernable. ^{45}Ca translocation from the root to shoot was driven mainly by the transpiration stream and xylem loading activity of ^{45}Ca was reflected. Plants treated under SD conditions for 3 months did not shed leaves and they remained green. However, ^{45}Ca translocation was obviously reduced in comparison with plants treated under LD or 3-week SD conditions (Fig. 4A-D). This ^{45}Ca decrease in the 3-month SD treatment suggests a gradual reduction in the transpiration stream under SD conditions. Transpiration stream reduction may be caused by ABA accumulation in the shoot, as is the case for apical buds (Rohde et al. 2002).

In contrast to the trend in shoot ^{45}Ca concentration, a 3-week SD treatment-specific tendency of decrease in root ^{45}Ca content occurred (Fig. 4A-D). This decrease was insignificant but might enhance the shoot to root ratio in ^{45}Ca allocation and likely indicates that the increase in Ca concentration in leaves after dormancy is attributable in part to this shoot-preferential distribution of Ca. However, considering the slight tendency of the translocation of the newly absorbed ^{45}Ca to the shoot tissue at the early dormancy, the drastic increases of Ca concentration in naturally grown leaves (Fig. 3) were suggested to be derived from the Ca loading into root xylem vessels using the root Ca absorbed before the onset of dormancy. Decreased Ca concentration in the root and increases in leaves in autumn also occur in naturally grown deciduous alder (*Alnus glutinosa*) (Rodríguez-Barrueco et al. 1984). The growth pattern of *P. maximowiczii* was categorized intermediate type between succeeding and flush types, on the other hand, *Alnus* sp. belonged succeeding and intermediate type, *Alnus hirsuta* and *Alnus japonica*, respectively (Kikuzawa 1983). Leaf emergence of succeeding type is one by one and long duration and flush type shows simultaneous leaf flush immediately after dormant bud break. The growth pattern and nutrient physiology are usually related (Killingbeck 1996, Noodén 2004), therefore, the activity of nutrient translocation from root to shoot might be

different between *P. maximowiczii* and alder. However, the Ca allocation shift that we observed might be one of the candidate mechanisms for the temporal decrease of Ca concentration in alder root. As indicated above, the driving force for Ca translocation to the shoot is the transpiration stream. However, the transpiration rate was not different between LD and SD treatments even though the daily period for active transpiration under LD conditions would have been longer. This suggests that, among others, one of the mechanisms explaining a change in the Ca shoot to root ratio is increased xylem Ca loading activity. Minerals absorbed by the root surface are transported to the vascular tissue and loaded into the xylem vessels. This activity is regulated by several mechanisms, but is mainly controlled by efflux transporters localized in vascular tissues. For example, Zn translocation from the root to the shoot is regulated by HMA4, a Zn efflux transporter expressed in root vascular tissues (Hanikenne et al. 2008). A gene encoding a Ca efflux transporter expressed in root vascular tissue and controlling Ca loading into the xylem vessels has yet to be identified. To fully explore these dormancy-induced mechanisms regulating Ca translocation, identifying the transporter for Ca xylem loading and analyzing gene expression in the dormancy process will be necessary.

In addition to the foregoing candidate mechanisms, the Ca concentration in xylem sap was also an important factor in Ca translocation from the root to the shoot. In xylem sap of beech (*Fagus sylvatica*), Ca concentration peaks from October to December and from March to April (Glavac et al. 1990). In our experiment, we did not observe active loading of newly absorbed Ca in plants shifted into deep dormancy (Fig. 4D). Because the growth pattern of *Fagus crenata* was categorized in flush type and different from intermediate type *P. maximowiczii* (Kikuzawa 1983), the Ca translocation activity might be also differed among them. Hence, for better understanding of Ca translocation, further investigations on the movement of Ca deposited in the apoplast and of Ca re-translocation should be conducted.

We showed that physiologically advantageous shifts in Ca allocation between shoots and roots and root Ca loading into xylem vessels occur in the early stage of dormancy. These dormancy-induced changes of root activity have not been reported before and the Ca translocation will be the model system for investigating root regulation at the onset of dormancy. To fully explore these dormancy-induced regulatory mechanisms in root function, molecular biological studies under artificially controlled conditions and verification of candidate mechanisms in genetically

manipulated plants will be required.

Acknowledgments

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