Seasonal Zinc Storage and a Strategy for Its Use in Buds of Fruit Trees

Ruohan Xie,a,b Jianqi Zhao,a,b Lingli Lu,a,b Patrick Brown,c Xianyong Lin,a,b Samuel M. Webb,d Jun Ge,a,b Olga Antipova,e Luxi Li,e and Shengke Tian,a,b,2,3

aMinistry of Education (MOE) Key Laboratory of Environment Remediation and Ecological Health, College of Environmental and Resource Science, Zhejiang University, Hangzhou 310058, China
bZhejiang Provincial Key Laboratory of Subtropic Soil and Plant Nutrition, Zhejiang University, Hangzhou 310058, China
cDepartment of Plant Sciences, University of California, Davis, California 95616
dStanford Synchrotron Radiation Lightsource, SLAC National Accelerator Laboratory, Menlo Park, California 94025
eAdvanced Photon Source, Argonne National Laboratory, Lemont, Illinois 60439

ORCID IDs: 0000-0001-6857-8608 (P.B.); 0000-0001-7706-7543 (J.G.); 0000-0001-8242-3581 (S.T.).

Bud dormancy allows deciduous perennial plants to rapidly grow following seasonal cold conditions. Although many studies have examined the hormonal regulation of bud growth, the role of nutrients remains unclear. Insufficient accumulation of the key micronutrient zinc (Zn) in dormant buds affects the vegetative and reproductive growth of perennial plants during the subsequent year, requiring the application of Zn fertilizers in orchard management to avoid growth defects in fruit trees. However, the mechanisms of seasonal Zn homeostasis in perennial plants remain poorly understood. Here, we provide new insights into Zn distribution and speciation within reproductive and vegetative buds during the winter season, and demonstrate that Zn stored in winter buds can be released for vegetative and reproductive growth during budbreak and subsequent meristematic growth. The mechanisms of Zn homeostasis during the seasonal cycles of plant growth and dormancy described here will contribute to improving orchard management, and to selection and breeding of deciduous perennial species.
In perennial deciduous trees, interruption or reduction of Zn availability during a given year will strongly affect vegetative growth and the success of new reproductive and vegetative development during the following year (Hu and Sparks, 1990; Swietlik, 2002). Thus, Zn deficiency in deciduous plants results in stunted growth, small leaves, and a rosette appearance at the shoot tip (Swietlik, 2002). This inhibition of shoot elongation and leaf development usually becomes visible in the early stages of spring growth after budbreak (Ojeda-Barrios et al., 2012; Xie et al., 2019), indicating that the emerging vegetative bud and early meristematic development have a very strong demand for Zn. Since spring budbreak occurs prior to active root growth and soil Zn uptake, deciduous plants must acquire and maintain an adequate Zn storage pool in buds for successful budbreak and initial growth (Maillard et al., 2015). Nevertheless, our understanding of the role of Zn bud storage in overwintering deciduous perennials remains limited.

The aforementioned significant gaps in our knowledge of plant biology are partly owing to a lack of information on the distribution of mineral nutrients within the bud. To date, research on the dynamic nutrient changes that occur during bud formation and budbreak has relied exclusively on whole bud analysis, which does not permit cellular localization of metals. To provide insight into the mechanisms whereby plants store and regulate Zn distribution during the seasonal cycles of growth and dormancy, it is essential to go beyond the quantification of total metal content in a bulk sample. Thus, here, we selected five species of deciduous fruit trees (apple [Malus domestica], peach (Amygdalus persica L.), grape (Vitis vinifera), pistachio (Pistacia vera), and blueberry [Vaccinium spp.]) to investigate the in vivo localization of Zn within terminal buds by application of microscopic and spectroscopic characterization techniques comprising synchrotron-based x-ray fluorescence (XRF) and x-ray absorption near-edge-structure (XANES) analyses. Most fruit crops are sensitive to Zn deficiency, and in many fruit orchards, especially in areas with calcareous and saline soils, Zn deficiency results in severe annual yield losses and deterioration of fruit quality (Swietlik, 2002). An examination of the spatial localization and the in vivo speciation of Zn in buds will provide a better understanding of the physiological processes responsible for Zn accumulation and the mechanisms of Zn supply during budbreak and early meristematic growth. This information will allow for improved management, selection, and breeding of deciduous perennial species.
RESULTS

Localization of Zn in Stem Nodes

Here we applied micro XRF (μ-XRF) analysis to investigate the localization of Zn in stem nodes in the upper, middle, and lower sections of apple-tree stems (Fig. 1). Our μ-XRF scans clearly showed that Zn was preferentially present in the vascular bundles at the point of stem-petiole connection (stem node). To further examine nutrient distribution patterns, we conducted spatial imaging of stem nodes having an axillary bud (Fig. 2A). In addition to Zn distribution in the vascular tissues of the stem node, axillary dormant buds also showed a remarkably pronounced localization of Zn, whose signal was detected in the axillary meristems protected by leaf primordia (Fig. 2B). In contrast, Zn concentration was much lower in the node-associated leaf, although the Zn signal was slightly elevated in the phloem of major leaf veins (Fig. 2C). The highest Zn concentration in the vascular bundles of stem nodes reached 0.4 μg cm⁻², which was much higher than that in the node-associated leaves (0.27 μg cm⁻²), suggesting that Zn was preferentially allocated to these nodes.

Analysis of Zn concentration in the internodes, nodes, and associated leaves confirmed these results (Fig. 3). Zn concentration was highest in the nodal parts of the lower section of stem nodes. Conversely, Zn concentration in the internodes was very low regardless of position. These results thus indicate that Zn preferentially distributes to the nodal regions of apple-tree stems.

Distribution and Speciation of Zn in Buds

Having identified the preferential allocation of Zn to stem nodes and axillary buds, we then examined the distribution and speciation of stored Zn in buds during winter (Figs. 4 and 5). We selected dormant terminal buds, rather than axillary buds, as experimental material for better structural observation and higher resolution of XRF scanning; further, by working with terminal buds, we avoided the risk of unsuccessful budbreak of axillary buds owing to apical dominance. Dormant vegetative buds consist of three major tissues: bud scales, leaf primordium, and pith with procambium tissue that will differentiate into xylem and...
phloem vessels to form vascular bundles. Zn preferentially allocated to the leaf primordium (Fig. 4). Then, we prepared transverse sections of terminal buds under high-pressure freezing (HPF) and analyzed these sections by nano-XRF to further reveal the details of Zn distribution at higher resolution. Figure 5A illustrates the incipient procambium region at the shoot apex of a dormant apple vegetative bud. Interestingly, there was a very strong association between the intensity of Zn and phosphorus (P) signals around the incipient procambium within cells. We acquired a second nano-XRF map from the transverse sections cut from near the base of the dormant bud (Fig. 5B). In XRF imaging, procambial tissue was particularly noticeable with a narrow band of closely arranged cells containing localized concentrations of Zn. Bands of xylem cells close to the procambium can be observed on the right-hand side of the image. As was observed in the terminal bud imagery (Fig. 5B), a close co-occurrence of Zn and P localization occurred in the intercellular spaces of procambium cells, whereas K predominantly localized in the cell walls of all types of cells within the mapped region (Fig. 5B). Higher-resolution elemental maps obtained from the incipient procambium region further revealed the presence of multiple, variously sized spots containing significant colocalization of Zn and P within incipient procambium cells. Statistical analysis revealed a significant linear correlation \( P < 0.05, r^2 = 0.516 \) between the intensity of Zn and P signals in this region (Fig. 5C). Based on the strong colocalization of Zn and P (Fig. 5, A–C), we inferred that Zn is likely chelated by P in the procambial cells. Further, the presence of Zn and P as “hotspots” suggested that P may be present in organic forms during seasonal storage.

We performed XANES analysis on powdered frozen-hydrated samples to acquire overall information about speciation of Zn in both dormant and vegetative buds during budbreak. Figure 5D shows the Zn K-edge, K3-weighted XANES spectra for bud tissues at the dormant and bud flush stages, and the spectra of eight model compounds. Linear-combination fitting revealed that Zn was present as Zn-phytate, Zn-nicotinamide, Zn-His, and Zn-polygalacturonic acid (Fig. 5D). During dormancy, Zn was present almost exclusively...
as Zn-phytate (88%), with smaller concentrations of Zn-His (12%). In contrast, during budbreak, Zn was present predominantly as Zn-nicotinamide (49%) and Zn-polygalacturonic acid (37%), while Zn-phytic acid decreased to 14.2% (Fig. 5E). These results indicated that phytate may play a role in the seasonal storage of Zn in dormant apple buds.

We further analyzed the concentration of different P fractions in buds/leaves during budbreak (Fig. 6). We selected four different budbreak stages (dormant bud,
swollen bud, bud flush, and new emerged leaves) for the measurement of total P (P$_{\text{tot}}$), inorganic phosphate (P$_i$), and organic phosphate (P$_{\text{org}}$). P$_{\text{tot}}$ was significantly enhanced after bud flush (Fig. 6A). P$_i$ was very low in dormant buds and increased significantly toward bud burst, with its maximum concentration during bud flush (Fig. 6B). In contrast, the concentration of P$_{\text{org}}$ in buds peaked in the dormant season and gradually declined after bud dormancy break (Fig. 6C). We also determined the variation in Zn concentration during budbreak. Zn level was relatively low during dormancy, increased significantly after bud flush in the early spring, and reached its highest concentration in the newly developing apple leaves (Fig. 6D).

Movement of Zn during Bud Development

To further explore the relationship between Zn and budbreak, we determined dynamic changes in stored Zn in terminal buds at different budbreak stages during winter and early spring (Fig. 7). We selected five budbreak stages (from dormant to total bud opening) for investigation (Fig. 7, A and B). XRF analysis showed a rapid budbreak-associated movement of Zn during bud development (Fig. 7C). At dormant stage, Zn preferentially distributed in the leaf primordium and, to a lesser extent, in two very narrow bands of the section that corresponds to the procambial strands (Fig. 7, dormant bud). During the initial stage of budbreak, the Zn signal decreased slightly around the leaf primordium, likely due to dilution by the growth and expansion of leaf primordia (Fig. 7, initiated development). During bud swelling, Zn localized in both leaf primordia and at a high concentration within procambial strands. These changes in localization may depend on the formation of functional vascular connections between bud and stem and the delivery of Zn via phloem tissue (Fig. 7, swollen bud and green tip). Following leaf emergence from the bud, Zn was most abundant in the vascular tissues subtending the emerged leaf (Fig. 7, bud flush). Preferential distribution of Zn in the vascular tissue was increasingly obvious after bud swell and especially pronounced at the bud flush stage (i.e. extended leaves visible), at which time the Zn signal in the vascular connection was ~2- to

Figure 6. P concentration in apple buds/leaves during budbreak. Concentrations of P$_{\text{tot}}$ (A), P$_i$ (B), P$_{\text{org}}$ (C), and Zn (D) in plant samples collected at four different budbreak stages. Data shown are means ± s of five biological replicates. Different lowercase letters indicate significant difference between samples collected from different stages at P < 0.05. DW, Dry weight.

Plant Physiol. Vol. 183, 2020 1205
Figure 7. Movement of Zn in an apple bud during budbreak. A and B, Microscope images show the morphology (A) and cross sections (B) of a bud at five different developmental stages. LP, Leaf primordium; Pr, procambium. C, Color-merge images show the relative location of Zn (red), potassium (K; blue), and calcium (Ca; green). D, Zn fluorescence intensity values were normalized for each map. Zn intensity values of the selected areas (marked with two white scanning lines) across the leaf primordium (L1) and vascular tissues (L2) are shown. Images were digitally extracted and made into a composite for comparison.
5-fold higher than in the same zone in buds at other developmental stages (Fig. 7D, L1 and L2). Conversely, the Zn signal in the leaf primordium peaked during the dormant stage and then remained steady after bud-break (Fig. 7D, L1 and L2).

**Buds Are a Valuable Seasonal Zn-Storage Site**

Here, we found that terminal buds and axillary buds are vital Zn storage sites in apple trees. To determine whether this is a common phenomenon among deciduous fruit species, we determined the Zn distribution patterns in dormant terminal buds collected from four other different deciduous fruit trees that represent most of the high-value fruit types (pome, drupe, berry, and nut; Fig. 8). The results confirmed that buds are high-density sites for mineral accumulation. Consistent with our observations in apple, leaf primordium and procambium within buds were preferential storage sites for Zn during dormancy in all four fruit species.

**DISCUSSION**

**Zn Preferentially Distributes in Stem Nodes**

The leaf-stem node is a crucial junction for the transfer of mineral nutrients to developing and dormant buds. A significant increase in Zn concentration was observed in nodal parts, specifically in the vascular tissues of stem nodes, indicating that nodes may act as a hub to reduce Zn flow for its preferential distribution to developing tissues and reproductive organs (Figs. 1–3). Additionally, we observed a node-based switch for the redistribution of Zn between xylem and phloem tissues (Fig. 1B). The transfer of Zn from the phloem in the lower stem to the xylem in the upper stem suggests that Zn is readily remobilized in apple trees and that some of the Zn in the older leaves can be remobilized to the new growing tissues. This finding is consistent with previous observations that suggest a high efficiency of the Zn phloem-transport system in some woody species (Tian et al., 2014; Saa et al., 2018; Xie et al., 2019). The potential intervascular transfer of Zn may also prolong the retention time of Zn, resulting in the enhanced Zn concentration observed in the node vascular tissues. This preferential distribution of Zn in the nodal parts (Figs. 2 and 3) is likely essential for the effective delivery of Zn to sink organs with high meristematic activity at times of limited vascular activity. The critical role of stem and reproductive nodes in the allocation of nutrients to reproductive structures has been previously described in rice (*Oryza sativa*), for which the nodes accumulate Zn to concentrations 10 times higher, or more than those in other tissues during both vegetative and reproductive stages. Various transporters localized at different cells in the vascular bundles also play important roles in the preferential distribution of nutrients in rice nodes (Yamaji et al., 2013; Yamaji and Ma, 2014, 2017).

**Zn Is Likely Sequestered as Phytate in Buds**

We applied HPF followed by freeze substitution for sample preparation, as previous studies have shown that this sample preparation protocol can preserve the in vivo localization of mobile elements (Smart et al., 2010; Moore et al., 2011). Here, we observed that Zn strongly colocalized with P inside procambium cells (Fig. 5, A–C). Most stored P in plants exists as P\textsubscript{org} (e.g. RNA, DNA, ATP, and membrane phospholipids), and ribosomal RNAs account for the largest P\textsubscript{org} pool (Ticconi and Abel, 2004; Péret et al., 2011; Lambers et al., 2015; Prodhan et al., 2019). Although phytic
acid (InsP6) accounts for a very small fraction of total P_{org}, it reportedly acts as the major phosphate storage compound in plant seeds and twigs (Brinch-Pedersen et al., 2002; Xue et al., 2007; Hu and Chu, 2017) and shows very high affinity for heavy metals in plants (Sarret et al., 2003; Bohn et al., 2008; Regvar et al., 2011; Kyriacou et al., 2014). Therefore, we hypothesized that Zn may exist as a phytate salt in primary procambium cells in the buds. Our study of the ligand environment of Zn at different budbreak stages confirmed this hypothesis (Fig. 5, D and E). Furthermore, the highest concentration of P_{org} observed in buds during dormancy suggested a seasonal storage of P in an organic compound in bud tissues. The increased concentration of P_{i}, combined with the gradual decline of P_{org} after budbreak, further indicated a transfer from P_{org} to P_{i} during budbreak (Fig. 6). This finding provides further evidence that Zn may be sequestered as Zn-phytate during bud dormancy and that this Zn-phytate is decationized and hydrolyzed under growth-promoting conditions.

While many studies have revealed the chelation of InsP6 with Zn in seeds (Lönnerdal, 2000; Urbano et al., 2000; Jiang et al., 2001; Ficco et al., 2009), the presence of Zn-phytate has not been previously reported in buds of deciduous trees. The occurrence of phytic acid in leaves is considered negligible, which makes it difficult to analyze its role as a Zn chelator in vegetative tissues. Although most plant cells, including those in vegetative tissues, can synthesize InsP6 (Raboy, 2003, 2009; Hadi Alkarawi and Zotz, 2014; Kurita et al., 2017), information about the presence and storage of InsP6 within plant leaves and vegetative buds is still limited. The formation of Zn phytate in leaves was previously reported only in some hyperaccumulator species or in circumstances in which plants were exposed to conditions of high exogenous Zn. The binding of Zn to phytic acid reportedly helps plants in limiting Zn mobility and reducing toxicity (Neumann and zur Nieden, 2001; Sarret et al., 2003; Dinh et al., 2015; Gupta et al., 2016; Doolittle et al., 2018). In poplar (Populus alba), InsP6 is abundant in overwintering twigs, where it is essential for long-term P storage in bark storage proteins together with various cations (calcium, magnesium, zinc, sodium, potassium, and lead); InsP6 rapidly decreased in early spring (Kurita et al., 2017). The decrease in speciation of Zn-phytate observed here (Fig. 5, D and E) suggests that this mechanism may be common for Zn reserves in apple buds during dormancy and that the accumulation of InsP6 at the very early stages of terminal bud formation may play an important role in seasonal Zn storage. At budbreak, InsP6 is likely decationized and hydrolyzed by phytases, followed by the release of P_{i}, inositol, and Zn.

After bud flush, a substantial proportion of Zn (48.7%) was contained in polygalacturonic acid, indicating that most of the Zn in newly emerged leaves is retained in the cell walls. Polygalacturonic acid is a major component of pectin (pectic polysaccharides), which is most abundant in plant primary cell walls and the middle lamella (Caffall and Mohnen, 2009). Various studies have shown that this major component of pectin in cell walls has a high binding capacity for Zn^{2+} (Khotimchenko et al., 2008) and thus limits the translocation of positively charged Zn^{2+} (Fernández and Brown, 2013; Doolittle et al., 2018). Our own results indicated an increase of Zn species as Zn-nicotianamine after bud flush, suggesting a possible positive role for nicotianamine-mediated Zn remobilization via the phloem tissues in apple trees. This observation is consistent with results of previous studies of a functional role for nicotianamine in long-distance transport of Zn in plants (Curie et al., 2009; Sinclair and Krämer, 2012; Xie et al., 2019).

### Zn Is Efficiently Released during Budbreak

Our results demonstrate that leaf primordium and procambium within buds are sites for high-density Zn storage. Both leaf primordium and procambium show very high levels of meristematic activity. The high level of Zn allocation in these tissues is consistent with the high Zn requirement of highly metabolically active differentiating cells, whereby it is more efficient for plants to store mineral elements needed directly at the growing point than to use energy for moving these elements from other storage locations when needed. The preferential distribution of Zn in the meristematic and vascular tissues has been observed in other plant tissues, such as in the plumule and radicle of germinating rice seeds (Wang et al., 2011; Lu et al., 2013a).

The dynamic spatial and temporal distribution of Zn described herein reflect the patterns of metabolism and development that would occur during bud germination.
and subsequent organ development. During bud development, Zn may be stored as Zn-phytate, which is then metabolized to provide Zn to the developing organs. Following the breaking of dormancy, new vascular connections form, and enhanced Zn delivery occurs (Fig. 7C). Some Zn derives from the relocation of Zn from the stem nodes, as seasonal storage of nutrients occurs in various perennial plant species. Woody plants must maintain an adequate storage pool for initial growth in the following spring, and nutrients are usually delivered from annual to perennial organs, such as stems or higher-order fine roots, to storage organs before dormancy (Estiarte and Peñuelas, 2015; Zadworny et al., 2015; Netzer et al., 2017). Secondly, Zn may come via direct root absorption. Overall, our data confirmed that a large amount of Zn is required at budbreak for specific growth-related functions, such as biosynthesis of protein and chlorophyll, enzyme activation (Coleman, 1998) and maintenance of membrane structure and functionality (Broadley et al., 2007; Palmgren et al., 2008). Further, Zn is required for the synthesis of auxin through its role in the biosynthesis of Trp (the amino acid precursor of auxin synthesis; Li et al., 2013). Recently, cross-talk between nutrients and auxin is receiving increasing attention. Some evidence seems to suggest that Zn-deficient plants show decreased auxin production, while other studies have shown that auxin signaling is closely associated with plant P status (Kobayashi et al., 2006; Rai et al., 2015; Begum et al., 2016; Sofo et al., 2017). Ferguson and Beveridge (2009) proposed that auxin is not in itself the internal cause of budbreak, but that auxin triggers budbreak by inducing the differentiation of the vascular system, thereby directing an adequate nutrient supply to the bud. The same authors found that the extent of sustained growth post-budbreak is limited by the ability of buds to continually attract nutrients (Ferguson and Beveridge, 2009). Other reports suggest that nutrients stimulate bud outgrowth by inducing the biosynthesis of hormones involved in the process (Umehara et al., 2010; Zhu and Kranz, 2012). In our study, spatial and temporal imaging of Zn demonstrated that budbreak precedes the development of new vascular connections (as indicated by new Zn flow into the buds) and that a highly efficient remobilization of Zn occurred during budbreak, which was accompanied by the differentiation of procambium into new functional vascular tissues. This is in agreement with the viewpoint that bud outgrowth, but not budbreak per se, is associated with the development of vascular connections to deliver nutrients where sink demand is high ( Husain and Linke, 1966; Ferguson and Beveridge, 2009). The findings described above warrant re-evaluation of the stimulating function of hormones in budbreak and plant development. Here, we propose that the presence of Zn in stem nodes is critical to provide Zn to developing buds and that this Zn supply is essential for budbreak and to support initial growth and development, which subsequently enhances vascular connections and nutrient flow to continue the nourishment of new developing organs.

This study reveals a likely universal adaptation strategy in deciduous perennials to store and regulate Zn flow during the seasonal cycles of growth and dormancy. As illustrated in Figure 9, during the active growth period, Zn flow is preferentially allocated to the stem nodes, which act as a hub to effectively redirect Zn to the developing buds to supply Zn for subsequent budbreak and development of new plant organs early in the following spring (Fig. 9A). Our data also indicate that Zn in the buds may be sequestered in the form of Zn phytate, a highly stable Zn storage pool. Subsequently, Zn phytate is used for budbreak and initial growth in the subsequent spring (Fig. 9B). During budbreak, Zn phytate is decationized and hydrolyzed, thus releasing Zn to trigger the initiation of budbreak and subsequent meristematic growth (Fig. 9C).

MATERIALS AND METHODS

Plant Material

Experiments were conducted on 5-year-old healthy apple trees (Malus domestica ‘Golden Delicious’) planted in a greenhouse at Zhejiang University (latitude 36°28' N; longitude 120°15' E). Each grafted tree was potted in a 20-L container filled with a mixture of soil:perlite (1:3) and watered with Hoagland nutrient solution as needed. Trees were grown under natural light supplemented with lamps (16 h/8 h photoperiod at 350 to 500 μmol m⁻² s⁻¹), under a 26°C/20°C day/night temperature regime and relative humidity 50% to 70%.

During the active growth stages, stem-petiole connections (nodes) were harvested to analyze Zn distribution patterns in stems at different developmental stages. At the end of winter, completely ecdormant apical buds were collected to investigate Zn seasonal storage status. To further study the dynamic nutrient status during budbreak, vegetative buds at different budbreak stages were selected for sampling, based on the previous determination of five stages of budbreak by macroscopic observation of bud morphology: dormant bud, initiated development, swollen bud (inner leaf scales visible), green tip, and bud flush (extended leaves visible). Four additional types of deciduous fruit trees, peach (Prunus persica), grape (Vitis vinifera), pistachio (Pistacia vera), and blueberry (Vaccinium spp.), were also sampled to investigate Zn distribution patterns in dormant terminal buds to determine whether the patterns observed in apple buds are common across deciduous fruit-tree species.

Mineral Element Mapping by μ-XRF and Nano-XRF

Samples for μ-XRF analysis were cut at a thickness of 180 μm using a cryotome (CM1950, Leica Biosystems) at −20°C (Tian et al., 2010). μ-XRF imaging was performed at the Stanford Synchrotron Radiation Laboratory using Beam Lines 2 and 3. Experiments were recorded at 13,500 eV, using a 20 × 20-μm beam spot size, 20 × 20-μm pixel size, and 100 ms dwell time per pixel. Fluorescence signal intensities for Zn, K, and Ca were calculated using SMAK software (http://www.ssrsl.slac.stanford.edu/~swebb/smak.htm). As it is rather difficult to obtain reliable spatial images of P in plant samples under the experimental conditions used here, P mapping was further addressed by using the Nano-XRF technique as described below. The Micro-XRF P-imaging results are shown in Supplemental Figure S1. The integrated intensities of Zn and other elements were calculated from XRF spectra and normalized by the intensity of the Compton scattering peak. Element mapping for the area measured was performed at the Stanford Synchrotron Radiation Laboratory using Beam

Plant Physiol. Vol. 183, 2020 1209

Seasonal Zinc Storage and Utilization in Buds
ultrastructure of samples and the in vivo spatial distribution of elements as intact as possible during sample processing and analysis. Nano-XRF was performed at a helium atmosphere on the Advanced Photon Source 2-ID-D and 2-ID-E hard x-ray microprobe beamline (Cai Z et al., 2003). Incident x-rays of 10 keV were chosen to excite elements from K to Zn. A Fresnel zone plate focused the x-ray beam to a spot size of 0.2 × 0.2 μm and 0.1 × 0.1 μm on the sample, which was raster-scanned at 1-μm step increments in the sample image, and 20 ms per pixel dwell time. Sample x-ray fluorescence was captured with an energy dispersive silicon drift detector. The resulting element maps were visualized and analyzed using MAPS (Vogt, 2003).

**Speciation of Zn measured by K-edge XANES**

Fresh, dormant and bursting apple buds were randomly sampled and ground into a powder for XANES analysis. Samples were prepared according to the method of Tian et al. (2011a). The methods used to prepare standard Zn compounds, including ZnSO4, Zn-citrate, Zn-His, Zn-nicotiamine, Zn-cell wall, Zn-polygalacturonic acid, and Zn-phytate, were based on previous studies (Kopittke et al., 2011; Lu et al., 2013b; Doolittle et al., 2018). XANES data were collected at the SSRL with the storage ring SPEAR-3 operating at 3 GeV and with ring currents of 80–100 mA. X-ray absorption spectroscopy of bulk tissues was carried out on SSRL beam lines 7-3 using the same equipment conditions described previously (Tian et al., 2010). Multiple scans (8–16, depending on Zn concentration) were collected and averaged for each sample to improve the signal-to-noise ratio. Principal component analysis and target transform were carried out using the SIXpack program. XANES data analysis was carried out using the Athena program suite according to standard methods (https://bruceravel.github.io/atom/). Spectra analysis was performed using linear combination fit analysis, which was carried out by calculating all possible component fits based on all reference spectra selected from principal component analysis and target transform. The best component fit was judged by the R-factor [sum(data-fit)/sum(data2)]. Best fits were derived by stepwise increase of the number of fit components and further optimized by minimizing fit residuals, which were defined as the normalized root-square difference between the data and the fit (Tian et al., 2011b).

**Measurement of Total Zn**

Leaves, internodes, and nodes were collected from the upper, middle, and lower sections of the experimental apple trees for Zn measurement. Samples were dried in an oven for 72 h until constant weight was reached, and dry weight was measured. The dried plant materials were ground, and 0.1-g samples were put into the glass digestion tubes. Then, 5.0 mL HNO3 was added, and samples were left overnight. The solution was then heated at 180°C using the graphite wall, Zn-polygalacturonic acid, and Zn-phytate, were based on previous studies (Kopittke et al., 2011; Lu et al., 2013b; Doolette et al., 2018). XANES data were collected at the SSRL with the storage ring SPEAR-3 operating at 3 GeV and with ring currents of 80–100 mA. X-ray absorption spectroscopy of bulk tissues was carried out on SSRL beam lines 7-3 using the same equipment conditions described previously (Tian et al., 2010). Multiple scans (8–16, depending on Zn concentration) were collected and averaged for each sample to improve the signal-to-noise ratio. Principal component analysis and target transform were carried out using the SIXpack program. XANES data analysis was carried out using the Athena program suite according to standard methods (https://bruceravel.github.io/atom/). Spectra analysis was performed using linear combination fit analysis, which was carried out by calculating all possible component fits based on all reference spectra selected from principal component analysis and target transform. The best component fit was judged by the R-factor [sum(data-fit)/sum(data2)]. Best fits were derived by stepwise increase of the number of fit components and further optimized by minimizing fit residuals, which were defined as the normalized root-square difference between the data and the fit (Tian et al., 2011b).

**Measurement of Different P Fractions**

Samples were dried in an oven for 72 h until constant weight was reached, and dry weight was measured. The dry, ground plant samples were ground into glass digestion tubes and 5.0 mL of 98% (v/v) H2SO4 was added. The solution was preheated at 180°C using the graphite digester and then digested at 280°C. Then 1 mL H2O2 was added several times until digestion was complete. The concentration of Zn in the filtrates was determined using inductively coupled plasma mass-spectroscopy (Agilent 7500a). The apple composition analysis certified reference materials (Aoke Biotechnology) was used as standard reference material.

**Statistical Analysis**

All data were statistically analyzed using the SPSS package (version 11.0). ANOVA was performed on the data sets. The mean and SE of each treatment were calculated, and Fisher’s LSD test was used to determine significant differences (P < 0.05 for significant and P < 0.01 for highly significant) for each set of data.

**Supplemental Data**

The following supplemental materials are available.

**Supplemental Figure S1.** Distribution patterns of P in apple buds during different bud break stages.

**ACKNOWLEDGMENTS**

We express our sincere gratitude to the staff of Beam Lines 2 and 3 at the Stanford Synchrotron Radiation Lightsource for their support. A part of this study was carried out at the Stanford Synchrotron Radiation Lightsource, a Directorate of the Stanford Linear Accelerator Center National Accelerator Laboratory and an Office of Science User Facility operated for the U.S. Department of Energy Office (DOE) of Science by Stanford University. The Stanford Synchrotron Radiation Lightsource Structural Molecular Biology Program is supported by the DOE Office of Biological and Environmental Research and by the National Institutes of Health, National Center for Research Resources, Biomedical Technology Program (grant no. P41RR01209). The use of the Advanced Photon Source, an Office of Science User Facility operated for the U.S. DOE by Argonne National Laboratory, was supported by the U.S. DOE (contract no. DE-AC02-06CH11357). The authors acknowledge the support of the Center of Cryo-Electron Microscopy, Zhejiang University, for sample preparation. We also thank Editage for English language editing.

Received March 24, 2020; accepted May 9, 2020; published May 18, 2020.

**LITERATURE CITED**

Alloway BJ (2009) Soil factors associated with zinc deficiency in crops and humans. Environ Geochim Health 31: 537–548

Begum MC, Islam M, Sarkar MR, Azad MAS, Huda AKMN, Kabir AH (2016) Auxin signaling is closely associated with Zn-efficiency in rice (Oryza sativa L.). J Plant Interact 11: 124–129

Bohn L, Meyer AS, Rasmussen SK (2008) Phytate: Impact on environment and human nutrition. A challenge for molecular breeding. J Zhejiang Univ Sci B 9: 165–191

Brinch-Pedersen H, Sørensen LD, Holm PB (2002) Engineering crops: Getting a handle on phosphate. Trends Plant Sci 7: 118–125

Broadley MR, White PJ, Hammond JP, Zelko I, Lux A (2007) Zinc in plants. New Phytol 173: 677–702

Caffall KS, Mohnen D (2009) The structure, function, and biosynthesis of plant cell wall pectic polysaccharides. Carbohydr Res 344: 1879–1900

Cai Z, Lai B, Xiao YSX (2003) An x-ray diffraction microscope at the advanced photon source. J Phys IV France 104: 17–20

Cakmak I (2008) Enrichment of cereal grains with zinc: Agronomic or genetic biofortification? Plant Soil 302: 1–17

Cline MG, Bhave N, Harrington CA (2009) The possible roles of nutrient deprivation and auxin repression in apical control. Trees (Berl West) 23: 489

Coleman JE (1998) Zinc enzymes. Curr Opin Chem Biol 2: 222–234

Considine MJ, Considine JA (2016) On the language and physiology of dormancy and quiescence in plants. J Exp Bot 67: 3189–3203

Curie C, Cassin G, Couch D, Divol F, Higuchi K, Le Jean M, Misson J, Schikora A, Czernic P, Mari S (2009) Metal movement within the plant: Contribution of nicotianamine and yellow stripe 1-like transporters. Annu Bot 103: 1–11

Dinh NT, Vu DT, Mulligan D, Nguyen AV (2015) Accumulation and distribution of zinc in the leaves and roots of the hyperaccumulator Noccaea caerulescens. Environ Exp Bot 110: 85–95

Doolittle CL, Read TL, Li C, Scheckel KG, Donner E, Kopittke PM, Schjoerring JK, Lombi E (2018) Foliar application of zinc sulphate and zinc EDTA to wheat leaves: Differences in mobility, distribution, and speciation. J Exp Bot 69: 4469–4481

Estiarte M, Peñuelas J (2015) Alteration of the phenology of leaf senescence and fall in winter deciduous species by climate change: Effects on nutrient productivity. Glob Change Biol 21: 1005–1017

Ferguson BJ, Beveridge CA (2009) Roles for auxin, cytokinin, and strigolactone in regulating shoot branching. Plant Physiol 149: 1929–1944

Fernández V, Brown PH (2013) From plant surface to plant metabolism: The uncertain fate of foliar-applied nutrients. Front Plant Sci 4: 289
Ficco DBM, Riesfico C, Nicastro G, De Simone V, Di Gesu AM, Beleggia R, Platani C, Cattivelli L, De Vita P (2009) Phytate and mineral elements concentration in a collection of Italian durum wheat cultivars. Field Crops Res 111: 235–242

Gregory FG, Veale JA (1957) A reassessment of the problem of apical dominance. Symp Soc Exp Biol 11: 1–20

Gupta N, Ram H, Kumar B (2016) Mechanism of zinc absorption in plants: Uptake, transport, translocation and accumulation. Rev Environ Sci Biotechnol 15: 89–109

Hadi Alkarawi H, Zotz G (2014) Phytic acid in green leaves. Plant Biol (Stuttgart) 16: 697–701

Horvath DP, Anderson JV, Chao WS, Foley ME (2003) Knowing when to grow: Signals regulating bud dormancy. Trends Plant Sci 8: 534–540

Hu B, Chu C (2017) Node-based transporter: Switching phosphorus distribution. Nat Plants 3: 17002

Hu HN, Sparks D (1990) Zinc-deficiency inhibits reductive development in ‘Stuart’ pecan. HortScience 25: 1392–1396

Husain SM, Linkn AJ (1996) Relationship of apical dominance to nutrient accumulation pattern in Pisum sativum var. Alaskan. Physiol Plant 19: 992–1010

Jiang L, Phillips TE, Hamm CA, Drozdzowicz YM, Rea PA, Maeshima M, Kopittke PM, Menzies NW, de Jonge MD, McKenna BA, Donner E, Paulsen RE, Díaz-Uriarte R, Bazzaz FA, Pineda LM, Morel F, Pérez R, Platani C, Cattivelli L, De Vita P (2009) Phytate and mineral elements concentration in the hyperaccumulator plant species Sedum alfredii. J Exp Bot 60: 3717–3730

Kbaryasai K, Masuda T, Takam Y, Ohta H (2006) Membrane lipid alteration during phosphate starvation is regulated by phosphate signaling and auxin/cytokinin cross-talk. Plant J 47: 238–248

Kopittke PM, Menzies NW, de Jonge MD, McKenna BA, Donner E, Webb RI, Paterson DJ, Howard DL, Ryan CG, Glover CJ, et al (2011) In situ distribution and speciation of toxic copper, nickel, and zinc in hydraulic conductors of cowpea. Plant Physiol 156: 663–673

Kurita Y, Baba K, Ohnishi M, Matsubara R, Kosuke K, Anegawa A, Shichijo C, Ishizaki K, Kaneko Y, Hayashi M, et al (2017) Inositol hexakis phosphate is the seasonal phosphorus reservoir in the deciduous woody plant Populus alba. Plant Cell Physiol 58: 1477–1485

Kyriacou B, Moore KL, Paterson D, de Jonge MD, Howard DL, Stangoulis J, Tester M, Lombi E, Johnsson B, et al (2014) Localization of iron in rice grain using synchrotron x-ray fluorescence microscopy and high resolution secondary ion mass spectrometry. J Cereal Sci 59: 173–180

Lambers H, Finnegan PM, Jost R, Plaxton WC, Shane MW, Stitt M (2015) Phosphorus nutrition in Proteaceae and beyond. Nat Plants 1: 15109

Leyser O (2003) Regulation of shoot branching by auxin. Trends Plant Sci 8: 441–445

Li Y, Zhang Y, Shi D, Liu X, Qin J, Ge Q, Xu L, Pan X, Li W, Zhu Y, et al (2013) Spatial-temporal analysis of zinc homeostasis reveals the response mechanisms to acute zinc deficiency in Sorghum bicolor. New Phytol 200: 1102–1115

Loeb J (1924) Regenration from a Physico-Chemical viewpoint. McGraw-Hill, New York

Lönnmarker B (2000) Dietary factors influencing zinc absorption. J Nutr 130(Suppl 737): 1378S–1388S

Lu L, Tian S, Liao H, Zhang J, Yang X, Labavitch JM, Chen W (2010) Analysis of metal element distributions in rice (Oryza sativa L.) seeds and relocation during germination based on x-ray fluorescence imaging of Zn, Fe, K, Ca, and Mn. PLoS One 5: e57360

Lu L, Tian S, Zhang J, Yang X, Labavitch JM, Webb SM, Latimer M, Brown PH (2013b) Efficient xylem transport and phloem remobilization of Zn in the hyperaccumulator plant species Sedum alfredii. New Phytol 198: 721–731

Maillard A, Diquelou S, Billard V, Lainé P, Garnica M, Prudent M, García-Mina JM, Yvin JC, Ourry A (2015) Leaf mineral nutrient re-mobilization during leaf senescence and modulation by nutrient deficiency. Front Plant Sci 6: 317

Martín-Fontera ES, Tarancón C, Cubas P (2018) To grow or not to grow, a power-saving program induced in dormant buds. Curr Opin Plant Biol 41: 102–109

Moore KL, Schröder M, Wu Z, Martin BG, Hawes CR, McGrath SP, Hawkesford MJ, Feng Ma J, Zhao FJ, Grovenor CR (2011) High-resolution secondary ion mass spectrometry reveals the contrasting subcellular distribution of arsenic and silicon in rice roots. Plant Physiol 156: 913–924

Netzer F, Schmid C, Herschbach C, Rennenberg H (2017) Phosphorus-nutrition of European beech (Fagus sylvatica L.) during annual growth depends on tree age and P-availability in the soil. Environ Exp Bot 137: 194–207

Neumann D, Neud N, Dieder Koonen U (2001) Silicon and heavy metal tolerance of higher plants. Phytochemistry 56: 685–692

Ojeda-Barrios D, Abadía J, Lombardini L, Abadía A, Vázquez S (2012) Zinc deficiency in field-grown pecan trees: Changes in leaf nutrient concentrations and structure. J Sci Food Agric 92: 1672–1678

Palmgren MG, Clemens S, Williams LE, Krämer U, Borg S, Schjerring JK, Sanders D (2008) Zinc biofortification of cereals: Problems and solutions. Trends Plant Sci 13: 464–473

Paul In, Hinke P, van der Schoot C (2014) Shoot meristems of deciduous woody perennials: Self-organization and morphogenetic transitions. Curr Opin Plant Biol 17: 86–95

Péret B, Clément M, Nussaume L, Desnos T (2011) Root developmental adaptation to phosphate starvation: Better safe than sorry. Trends Plant Sci 16: 442–450

Prodhann MA, Finnegan PM, Lambers H (2010) How does evolution in phosphorus-impoeverished landscapes impact plant nitrogen and sulfur assimilation? Trends Plant Sci 24: 69–82

Raboy V (2003) myo-Inositol-1,2,3,4,5,6-hexakisphosphate. Phytochemistry 64: 1033–1043

Raboy V (2009) Approaches and challenges to engineering seed phytate and total phosphorus. Plant Sci 177: 281–296

Rai V, Sanagala R, Siniall B, Yadav S, Sarkar AK, Danuk PK, Jain A (2015) Iron availability affects phosphate deficiency-mediated responses, and evidence of cross-talk with auxin and zinc in Arabidopsis. Plant Cell Physiol 56: 1107–1123

Regvar M, Eichert D, Kaulich B, Gianoncelli A, Pongrac P, Vogel-Mikus R, Platani C, Cattivelli L, De Vita P (2009) Phosphorus mobilization during leaf senescence and modulation by nutrient deficiency in the accumulator Arabidopsis thaliana grown in cadmium- and zinc-enriched media. J Plant Physiol 216: 174–180

Saa S, Negron C, Brown P (2018) Folic acid zinc applications in Prunus: From lab experience to orchard management. Sci Hortic (Amsterdam) 233: 233–237

Sarret G, Saamitou-Laprade P, Bert V, Proux O, Hazemann JL, Traverse P, Sinclare SA, Kramer U (2003) Forms of zinc accumulated in the hyperaccumulator plant species Sedum alfredii hyperaccumulator plant species. Plant Physiol 130: 1815–1826

Sinclair SA, Krämer U (2012) The zinc homeostasis network of land plants. Biochim Biophys Acta 1825: 1553–1567

Smart KE, Smith JA, Kilburn MR, Martin BG, Hawes C, Grovenor CR (2010) High-resolution elemental localization in vacuolate plant cells by nanoscale secondary ion mass spectrometry. J Plant Physiol 167: 870–879

Sofo A, Bochichio R, Amato M, Rendina N, Vitti T, Nuzzaci M, Altamura MM, Falasca G, Rovese FD, Scopa A (2017) Plant architecture, auxin homeostasis and phenol content in Arabidopsis thaliana grown in cadmium- and zinc-enriched media. J Plant Physiol 216: 174–180

Swiekel D (2002) Zinc nutrition of fruit crops. Horttechnology 12: 45–50

Tian S, Lu L, Labavitch J, Yang X, He Z, Hu H, Sarangi R, Newille M, Comisso J, Brown P (2011a) Cellarular sequestration of cadmium in the hyperaccumulator plant species Sedum alfredii. Plant Physiol 157: 1914–1925

Tian S, Lu L, Labavitch JM, Webb SM, Yang X, Brown PH, He Z (2014) Spatial imaging of Zn and other elements in Huanglongbing-affected grapefruit by synchrotron-based micro x-ray fluorescence investigation. J Exp Bot 65: 953–964

Tian SK, Lu LL, Yang XE, Huang HG, Brown P, Labavitch J, Liao HB, He ZL (2011b) The impact of EDTA on lead distribution and speciation in the accumulator Sedum alfredii by synchrotron X-ray investigation. Environ Pollut 159: 782–788

Tian S, Lu L, Yang X, Webb SM, Du Y, Brown PH (2010) Spatial imaging and speciation of lead in the accumulator plant Sedum alfredii by microscopically focused synchrotron X-ray investigation. Environ Sci Technol 44: 5920–5926

Ticconi CA, Abel S (2004) Short on phosphate: Plant surveillance and countermeasures. Trends Plant Sci 9: 548–555
Xie et al.

Umehara M, Hanada A, Magome H, Takeda-Kamiya N, Yamaguchi S (2010) Contribution of strigolactones to the inhibition of tiller bud outgrowth under phosphate deficiency in rice. Plant Cell Physiol 51: 1118–1126

Urbano G, López-Jurado M, Aranda P, Vidal-Valverde C, Tenorio E, Porres J (2000) The role of phytic acid in legumes: Antinutrient or beneficial function? J Physiol Biochem 56: 283–294

Vogt S (2003) MAPS: A set of software tools for analysis and visualization of 3D x-ray fluorescence data sets. J Phys IV France 104: 635–638

Wang YX, Specht A, Horst WJ (2011) Stable isotope labelling and zinc distribution in grains studied by laser ablation ICP-MS in an ear culture system reveals zinc transport barriers during grain filling in wheat. New Phytol 189: 428–437

Xie R, Zhao J, Lu L, Ge J, Brown PH, Wei S, Wang R, Qiao Y, Webb SM, Tian S (2019) Efficient phloem remobilization of Zn protects apple trees during the early stages of Zn deficiency. Plant Cell Environ 42: 3167–3181

Xue H, Chen X, Li G (2007) Involvement of phospholipid signaling in plant growth and hormone effects. Curr Opin Plant Biol 10: 483–489

Yamaji N, Ma JF (2014) The node, a hub for mineral nutrient distribution in graminaceous plants. Trends Plant Sci 19: 556–563

Yamaji N, Ma JF (2017) Node-controlled allocation of mineral elements in Poaceae. Curr Opin Plant Biol 39: 18–24

Yamaji N, Sasaki A, Xia JX, Yokosho K, Ma JF (2013) A node-based switch for preferential distribution of manganese in rice. Nat Commun 4: 2442

Zadworny M, McCormack ML, Rawlik K, Jagodziński AM (2015) Seasonal variation in chemistry, but not morphology, in roots of Quercus robur growing in different soil types. Tree Physiol 35: 644–652

Zhou J, Jiao F, Wu Z, Li Y, Wang X, He X, Zhong W, Wu P (2008) OsPHR2 is involved in phosphate-starvation signaling and excessive phosphate accumulation in shoots of plants. Plant Physiol 146: 1673–1686

Zhu H, Kranz RG (2012) A nitrogen-regulated glutamine amidotransferase (GAT1_2.1) represses shoot branching in Arabidopsis. Plant Physiol 160: 1770–1780