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ORIGINAL ARTICLE

S-ABA-induced changes in root to shoot partitioning of root-applied ^{44}Ca in apple (*Malus domestica* Borkh.)

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Abstract

Key message **Using ^{44}Ca as a stable isotope tracer, calcium transport to aboveground portions of apple trees was associated with transpiration rates, but overall calcium uptake was not.**

Calcium is a critical plant nutrient with important roles in quality, storability of fruit, and resistance to abiotic and biotic stresses. Calcium is largely immobile in the plant, increasing the risk of localized calcium deficiencies. It can be difficult to quantify how changes in transpiration affect calcium uptake and allocation to aboveground organs. Here, the effect of an exogenous abscisic acid (ABA) application on calcium uptake by roots and allocation to leaves of ***Malus domestica*** Borkh. cv. Honeycrisp was measured by isotopically labeling the potting media with ^{44}Ca and measuring tracer movement after 30 days. Gravimetric water use and leaf level transpiration were lower in trees that were treated with ABA. ABA application reduced water use by more than 40% immediately after treatment and its effect was sustained. Uptake of ^{44}Ca into the plant was not significantly different between ABA-treated trees and untreated trees, but calcium allocation between roots and shoots was affected. The amount of ^{44}Ca tracer in shoots after 30 days was lower in ABA-treated trees compared to the untreated control and corresponded to a greater proportion of ^{44}Ca in the roots, suggesting that calcium allocation to aboveground parts is dependent on transpiration but calcium uptake by the roots is not.

Keywords ^{44}Ca · Transpiration · Calcium allocation · Calcium uptake

Introduction

Calcium is an essential element to plant form and function (Gilliham et al. 2011; de Freitas et al. 2012; Marschner 2012; Hocking et al. 2016). In many agricultural crops, it is a critical factor contributing to quality, storability of fruit, and resistance to abiotic and biotic stress. Calcium contributes to the structural integrity of cell walls and is a critical messenger for plant responses to environmental cues (Kudla et al. 2010; Batistič and Kudla 2012). In fleshy horticultural crops, localized deficiencies in calcium can cause decrease in fruit quality and increase in storage disorders that can result in significant losses to the agricultural industry (Bangerth 1973, 1979; Shear 1975; Simon 1978; White and Broadley 2003). Localized cracking, senescent breakdown or other fruit issues were the most common disorders, but leaf-based disorders have also been attributed to calcium. These postharvest disorders include blossom end rot in *Lycopersicon esculentum* Mill. (tomato), bitter pit in *Malus domestica* Borhk. (apple), cork spot in *Pyrus communis* L. (pear), and leaf burn in *Lactuca sativa* L. (lettuce) (White and Broadley 2003). While this phenomenon has been intensively studied and described in many crop plants for the past century (Bangerth 1973, 1979; Shear 1975; Simon 1978; Vang-Petersen 1980; Ferguson and Watkins 1989), the underlying mechanisms regulating the development of these disorders and strategies that stimulate calcium delivery to aboveground tissues have not been identified. In these reports, analogous calcium-related disorders can occur across many plant species in response to soil-based deficiencies but, in general, symptoms were often expressed independent of soil calcium availability.

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Calcium uptake occurs at the root surface and translocation to the shoots occurs via the xylem stream (Hanger 1979). This is the first critical step of ensuring sufficient calcium delivery to transpiring plant tissues. Root uptake occurs via the apoplastic pathway as a divalent cation through the mass flow of water (Barber 1995). However, for plant species that develop a Casparian band, at some point, uptake must transition to symplastic transport prior to xylem loading and transport to the growing shoot (White 2001; Gilliham et al. 2011). Xylem transports and unloads mineral-laden water to sink organs like leaves and fruit. Unlike other nutrients such as potassium and nitrogen, calcium is not mobile in the phloem (Wiersum 1966; Epstein 1973; Hanger 1979; Gilliham et al. 2011; Hocking et al. 2016). Once calcium has been removed from the xylem stream, localized deficiencies within the plant cannot stimulate the redistribution of calcium. Calcium allocation thus depends on the flow of water through the transpiration stream (Plamboeck et al. 2000; Jonard et al. 2009), though recent studies have shown that ion exchange processes in the xylem greatly influence the velocity of calcium transport (van der Heijden et al. 2015; Schmitt et al. 2017). In general, fruit have lower transpiration rates than leaves (Lang 1990; Montanaro et al. 2006, 2014; Hocking et al. 2016). Therefore, calcium delivery will preferentially be weighted towards the leaves. Greater calcium accumulation has been reported in leaves compared to fruit during the growing season (Clark et al. 1987). Several factors can alter calcium allocation to different aboveground organs. Montanaro et al. (2014) demonstrated that a decrease in fruit transpiration can limit the allocation of calcium to the fruit, presumably through the reduction in xylem water delivery. Alternatively, de Freitas et al. (2014) reported a decrease in leaf calcium concentration and an increase in fruit calcium concentration in tomato plants treated with abscisic acid (ABA) relative to an untreated control. Similarly, changes in the transpiration rates of whole canopies may affect calcium distribution from the roots to aboveground organs. While water flow appears to impact calcium delivery from the roots to the aboveground portion of the plant, the capacity to take up calcium from the soil solution is equally important.

In the past century, radioisotope and stable isotope tracers have been used to study nutrient uptake and distributions in soil and plants (Nishigaki 1962). Some radioisotopes are versatile, inexpensive, and easily used research tools available in biological research. The measurement of calcium uptake by plants using an isotope tracer was traditionally done using a radioactive ^{45}Ca tracer. Wiersum (1966) used ^{45}Ca radioisotopes to study calcium movement in several horticultural species. Biddulph et al. (1958) measured ^{45}Ca uptake in bean plants and Chiu and Bould (1976) measured ^{45}Ca uptake in tomato. More specifically, Shear and Faust (1970) applied ^{45}Ca radioisotope to roots of apple seedlings to trace the translocation of calcium to aboveground portions. Shear and Faust (1970) reported the presence of radiotracer in the leaves of apple seedlings in as little as 3 days. Using a radiograph, the presence of the radioisotope was easily detected and imaged within plant tissue. However, past experiments were usually limited to greenhouses or lab environments because of radioactivity. Currently, potential health and environmental hazards frequently limit the use of radioisotopes to lab experiments (Rayburn 1990).

There are also stable calcium isotopes that can be used to trace movement and transformation. At natural abundance, total calcium consists of 2.086% ^{44}Ca and 96.941% ^{40}Ca (Hoefs 2009). To produce a signal that can be traced from the soil to different locations in the plant, the use of heavily enriched stable isotope tracers is often required. Similar to the use of other stable isotopes (^{15}N, ^{18}O, ^{13}C, ^{2}H), ^{44}Ca can be used to monitor calcium uptake and allocation in plants in less controlled environments than ^{45}Ca radioisotopes. The ^{44}Ca isotope has been used to trace biogeochemical fluxes and processes in terrestrial ecosystems (Page et al. 2008; van der Heijden et al. 2013; Fantle et al. 2014) and also in plants (van der Heijden et al. 2015; Schmitt et al. 2017). These approaches have the potential for further use in physiological studies exploring the dynamics of calcium uptake and allocation in plants.

Although there is strong evidence that differences in transpiration between leaves and fruit can alter the distribution of calcium delivery between these two sink organs, the impact of whole plant transpiration on overall calcium root uptake and allocation is less clear. In this study, we used the stable isotope ^{44}Ca tracer to understand the relationship between plant transpiration and calcium uptake and allocation from roots to shoots in non-fruiting apple trees. To do this, ^{44}Ca was used to quantify calcium uptake from the soil solution and allocation to leaves in an experimental system where whole plant transpiration was manipulated through exogenous ABA applications to the leaves. The results from this experiment provide insight into how leaf transpiration rates can affect calcium uptake dynamics and the allocation of calcium to below and aboveground plant parts.

Materials and methods

Plant material and experimental design

For the experiment, *Malus domestica* Borkh. cv. 'Honeycrisp' trees were grafted onto M9-T337 dwarfing rootstocks and grown for 2 years in a nursery. Thirty trees were selected from bare-root trees stored in cold storage at 2 °C. Trees were screened for uniformity and were approximately 1.8 m in height with a trunk caliper of 12 mm at 15 cm from the graft union. The trees were potted into 10 L pots with

approximately 1820 g of peat moss and perlite as a potting media. The pH of the potting media was 6.5. After bud break, the flower buds were removed to prevent any fruit formation.

Growing conditions and experimental design

The trees were grown in a greenhouse maintained at 23–25 °C with approximately 20–50% relative humidity for 60 days before the start of the experiment. The glass was covered with whitewash under ambient light conditions during June and July 2015. During this time, the mean daily maximum photosynthetically active radiation in the greenhouse was between 1000 and 1200 µmol m^{-2} day^{-1}. The experiment was carried out in July and August 2015. The mean total daily radiation during the experimental period, taken from the Washington State University Agricultural Weather Network monitoring station (http://www.weather.wsu.edu) outside the greenhouse ranged from 10 to 28 MJ m^{-2} day^{-1} (Fig. 1). Plants were watered daily to replace the amount of water lost through transpiration to maintain a consistent volumetric water content. The trees were fertilized weekly with a 1/10th strength modified Hoagland's solution (1.5 mM nitrogen, 600 µM potassium, 500 µM calcium, 100 µM phosphorous, 200 µM sulfur, 200 µM magnesium, and micronutrients: 5 µM chlorine, 2.5 µM boron, 0.2 µM manganese, 0.2 µM zinc, 0.05 µM copper, and 5 µM ferrous iron).

Treatments and experimental design

To start the experiment, trees were arranged in a completely randomized design and moved daily to prevent any positional effects in the greenhouse. After 60 days of growth, five randomly labeled trees were sprayed with 500 mg L^{-1} ABA (Protone, Valent Biosciences, Libertyville, IL) and another five were treated with a distilled water control. Each tree received approximately 100 mL of spray and was wet until spray dripped off of the leaves. The next day, once the effect of ABA on plant transpiration had been confirmed, 20 mg $^{44}CaCO_3$ per pot was added in the form of 97 atom% $^{44}CaCO_3$ to the potting media after being solubilized in 1 L of ultrapure water. Another five trees were treated with 20 mg of $CaCO_3$ at natural abundance to measure non-enriched plants. Care was taken to reduce the leaching of solution from the bottom of the pots and saucers were placed under each pot to capture any excess water/treatment solution. The pots were covered with reflective foil to prevent the loss of water through evaporation and to keep the roots from overheating. In this way, water loss only occurred through whole plant transpiration.

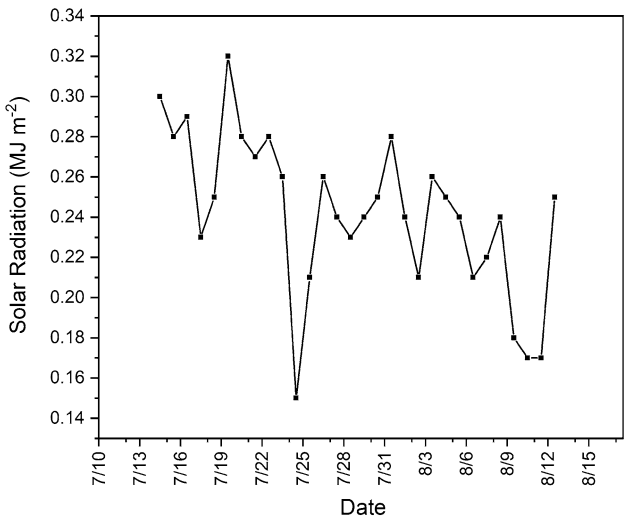

Fig. 1 Light intensity (MJ m^{-2}) recorded in Wenatchee, WA from July 15 to August 14, 2015

Gravimetric and gas exchange measurements of transpiration

Plant water use was measured both gravimetrically and using a leaf-specific gas exchange system. Leaf measurements were made 5 days prior to ABA applications, 2 days after ABA treatment, and 9 days after ABA treatments. These days were chosen to make comparisons between leaf and whole plant transpiration before and after treatments, while the gravimetric measurements were made to track water use over the duration of the experiment. Gravimetric plant water use for each pot was measured daily using an Ohaus ES30R portable field balance (Mettler Toledo, Columbus, OH). Pots were weighed between 09:00 and 10:00 before and after watering. Plants were watered to replace the amount of water that had been lost during the day. Daily water use was calculated as the difference between the weight of the pot after watering and the weight of the pot 24 h later prior to watering. Photosynthesis and transpiration were measured on three mature, sun-exposed leaves from each tree with a Li-Cor 6400XT (LiCor, Lincoln, NE) using the transparent leaf chamber at ambient light. Temperature was regulated at 25 °C and the vapor pressure deficit (VPD) was maintained at approximately 2.0 with flow rates of 400 µmol air m^{-2} s^{-1}. Leaves were placed in the chamber and photosynthetic rates were allowed to stabilize for 5 min. Once photosynthetic rates were stable, three recorded measurements were made 5 s apart and the average photosynthetic rate was taken as the value for each leaf. Transpiration and leaf photosynthetic rates were analyzed using a one-way ANOVA ($\alpha = 0.05$) in SAS (SAS Institute, Cary, NC) with ABA treatment as the fixed effect. Tukey's HSD test was used for mean separation of treatment. To compare gravimetric and individual leaf water use and account for variation in leaf area among

trees, whole tree water use was divided by the total leaf area destructively measured at the end of the experiment.

Plant and potting media sampling

After 30 days of treatments, plants were separated into leaves, stems, and roots. Potting media samples were also collected from each pot for total calcium and ^{44}Ca analysis. Plant material was air dried at 40 °C for 1 month. Whole samples were homogenized and then a 5 g subsample was placed in a 50 mL vial with stainless steel beads and ground to micron size using a VWR high throughput homogenizer (Product number:, VWR, Radnor, PA). A 200 ± 1 mg sample of ground tissue was weighed into digestion vials and hot plate digested with 6 mL of HNO_3. After the digestion was complete, the digest was filtered with a 0.45 µM PTFE filter (Product number 28145-493, VWR, Radnor, PA). Filtered digests were then diluted 100× to be used for elemental analysis. For potting media, exchangeable calcium was extracted from 2 g of potting media using 30 mL 1 M ammonium acetate buffered to a pH of 7.0 in a 50 mL conical tube. After the addition of ammonium acetate to the potting media, the mixture was shaken at 180 oscillations per minute and then gravity filtered for elemental analysis using Whatman 1 filter paper (GE Healthcare Science, Pittsburg, PA).

Chemical and isotope ratio analysis

Calcium concentrations of digests and ammonium acetate extractions were measured at Washington State University soil chemistry service laboratory using an Agilent 4200 microwave plasma absorption emissions spectrometer (MP-AES) (Agilent Technologies Inc., Santa Clara, CA) or at the INRA-BEF laboratory by inductively coupled plasma atomic emission spectroscopy (ICP-AES) on an Agilent 7500 series spectrometer (Agilent Technologies Inc., Santa Clara, CA). The $^{44}Ca/^{40}Ca$ isotope ratios in potting media, roots, stems, and leaves were measured with ICP-MS on an 820MS Analytical Jena ICP-MS (Analytik Jena AG, Jena, Germany) following isotope analysis methods (van der Heijden et al. 2013, 2015)_ENREF_24. The 820MS instrument is equipped with a collision/reaction cell. To eliminate the ^{40}Ar interference to measure ^{40}Ca, a H_2 reaction gas is used: H_2 is injected directly into the plasma (100 mL min-1); the H_2 gas reacts with Ar^+ ions and forms a neutral species (no electric charge) which does not enter the mass spectrometer portion of the instrument. Because the response of the detector to sample concentration is not linear, all samples were analyzed for calcium concentrations initially and then diluted to 100 µg $Ca{\cdot}L^{-1}$ prior to the ICP-MS analysis. Instrument mass bias is corrected for using the standard bracketing technique. Because the response of the detector is not linear with the ^{44}Ca enrichment of samples, calcium mass bias is corrected by inserting both the NIST 915b standard and an in-house ^{44}Ca-enriched standard ($\delta^{44}Ca = 1880\%$) in every 12 samples. Van der Heijden et al. (2013) determined the precision, repeatability, and accuracy of the ICP-MS methods as 3.2, 3.7 and 1.2%, respectively, for $^{44}Ca/^{40}Ca$. Methods were validated by instrument intercalibration (van der Heijden et al. 2013). Measured isotopic compositions of samples are expressed in permil deviations relative to the calcium reference ratios (NIST SRM 915a):

$$\delta^{44/40}Ca = \left\{ \left(^{44}Ca/^{40}Ca\right)_{sample} / \left(^{44}Ca/^{40}Ca\right)_{NIST915a} - 1 \right\} \times 1000$$

The µg $^{44}Ca_{potting\ media}$, µg $^{44}Ca_{roots}$, µg $^{44}Ca_{stems}$, and µg $^{44}Ca_{leaves}$ are the amount of Ca in those separate components that originate from the isotopic tracer added to the potting media, respectively. The µg $^{44}Ca_{potting\ media}$, µg $^{44}Ca_{roots}$, µg $^{44}Ca_{stems}$, and µg $^{44}Ca_{leaves}$ were calculated using the following equation:

$$mg^{44}Ca_i = \left(\frac{\alpha_i - \alpha_{control}}{\alpha_{tracer} - \alpha_{control}} \right) \times [Ca]_i \times mass_i$$

where, α_i is the ^{44}Ca isotopic composition of the potting media, roots, stems, or leaves of isotopically labeled plants (^{44}Ca atom%), $\alpha_{control}$ is the $^{44}Ca/^{40}Ca$ isotopic ratio of the untreated samples taken at the same time (^{44}Ca atom%), α_{label} is the $^{44}Ca/^{40}Ca$ isotopic ratio of the tracing solution (97 atom% ^{44}Ca), $[Ca]_i$ is the Ca concentration ($mg.g^{-1}$) and $mass_i$ is the dry mass of each calcium pool (mg).

α values are calculated from the measured $^{44}Ca/^{40}Ca$ isotopic ratio as follows:

$$\alpha = \frac{^{44}Ca/^{40}Ca}{1 + {}^{42}Ca/^{40}Ca + {}^{43}Ca/^{40}Ca + {}^{44}Ca/^{40}Ca + {}^{46}Ca/^{40}Ca + {}^{48}Ca/^{40}Ca}$$

where $^{44}Ca/^{40}Ca$ is the measured ratio, $^{42}Ca/^{40}Ca$, $^{43}Ca/^{40}Ca$, $^{46}Ca/^{40}Ca$, and $^{48}Ca/^{40}Ca$ were assumed to be constant and equal to terrestrial values: 6.677×10^{-3} for $^{42}Ca/^{40}Ca$, 1.3926×10^{-3} for $^{43}Ca/^{40}Ca$, 4.1262×10^{-5} for $^{46}Ca/^{40}Ca$, and 1.929×10^{-3} for $^{48}Ca/^{40}Ca$ (Hoefs 2009).

Then, the total recovery of ^{44}Ca tracer in the potting media and the plant was calculated as:

$$\% \text{ recovery} = \left(\frac{mg^{44}Ca_{\text{potting media}} + mg^{44}Ca_{\text{roots}} + mg^{44}Ca_{\text{stem}} + mg^{44}Ca_{\text{leaves}}}{8 \text{ mg}} \right) \times 100\%$$

Total shoot uptake was calculated as the sum of leaf and stem tracer recovery (mg) and total plant uptake was the sum of tracer recovery for all plant parts and was calculated as follows:

$$\text{total plant uptake (mg)} = mg^{44}Ca_{\text{potting media}} + mg^{44}Ca_{\text{roots}} + mg^{44}Ca_{\text{stem}} + mg^{44}Ca_{\text{leaves}}$$

The percent plant uptake of applied tracer was calculated as:

$$\% \text{ plant uptake} = \frac{\text{total plant uptake (mg)}}{8 mg^{44}Ca \text{ applied}}$$

Statistical analysis

Data were analyzed using SAS (SAS Institute, Cary, MC) for one-way analysis of variance. Linear regression was performed for testing the relationship between light intensity and transpiration using OriginPro ($\alpha = 0.05$).

Results

Plant water use declined immediately upon ABA application. Water use was reduced by approximately 40% by the application of ABA. The difference in water use between the ABA-treated trees and the untreated control decreased with time (Fig. 2). Gravimetrically determined plant water use for the control trees ranged from 296 to 638 mL day^{-1}. For trees treated with ABA, the daily plant water use ranged from 90 to 358 mL day^{-1}. Plant water use was significantly correlated with daily light intensity (Fig. 3) where water use was lower for days with decreased light intensity. Additionally, the slope of the lines for ABA-treated trees and trees from the untreated control were significantly different ($P < 0.05$).

Prior to treatment, stomatal conductance (G_s) was not significantly different between ABA-treated trees and the untreated control (Fig. 4). As expected, after the application of exogenous ABA as a foliar spray, stomatal conductance decreased by more than 60% compared to the control. Plant water use measured at the leaf level also decreased for trees treated with ABA, indicating that reductions in water use were linked to a decrease in stomatal conductance. Prior to treatment, leaf transpiration (E) measured with a Li-Cor 6400XT was 3.8 and 3.7 mmol H_20 m^{-2} s^{-1} in untreated trees and ABA-treated trees, respectively (Fig. 4). After treatment, transpiration decreased to 1.2 mmol H_2O m^{-2} s^{-1} for ABA-treated trees and remained unchanged for the untreated control. Even 9 days after application, transpiration was still significantly lower in ABA-treated trees compared to untreated trees (Fig. 4). Leaf level transpiration

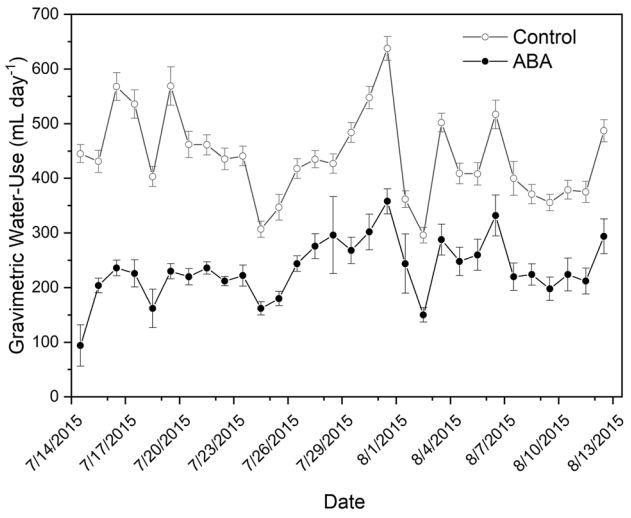

Fig. 2 Daily transpiration measured through gravimetric measurements of ABA-treated (closed circles) and control (open circles) 'Honeycrisp' apple trees measured over 30 days; error bar represent the standard error of the mean ($N = 5$)

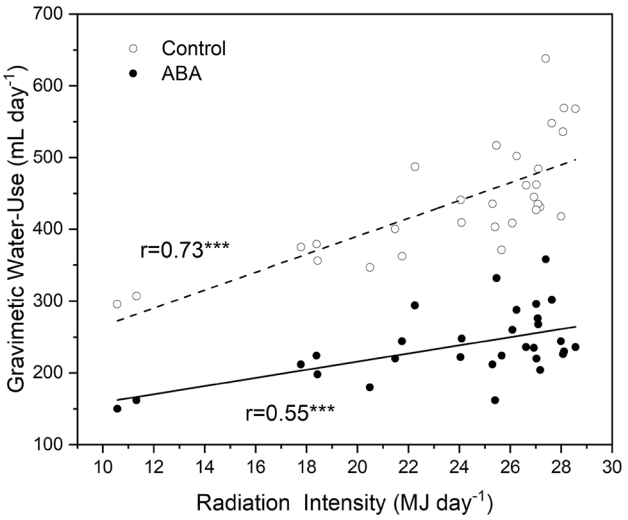

Fig. 3 The relationship between daily outdoor light radiation (MJ m^{-2}) and whole plant transpiration (mL day^{-1}) for 'Honeycrisp' apple treated with either ABA (closed circles) compared to an untreated control (open circles) over 30 days; lines represent best linear regression fit for either ABA-treated trees (solid line) or an untreated control (dashed line). ***$P < 0.001$

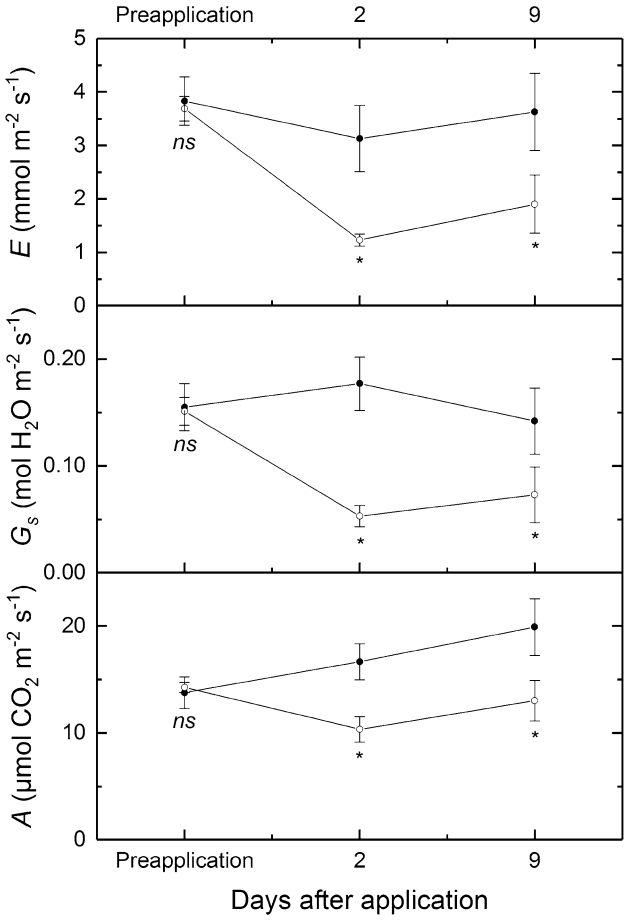

Fig. 4 Mean photosynthetic rate (A, µmol CO_2 $m^{-2}s^{-1}$), stomatal conductance (G_s, mol H_2O $m^{-2}s^{-1}$), and transpiration rates (E, mmol H_2O $m^{-2}s^{-1}$) of ABA-treated (open circles) and control 'Honeycrisp' apple trees (solid circles) before, 2 and 9 days after treatment measured using a LiCor 6400XT gas exchange system (Mean ± SE; $N = 5$). *Significant differences between means determined using a Tukey's HSD test ($\alpha = 0.05$)

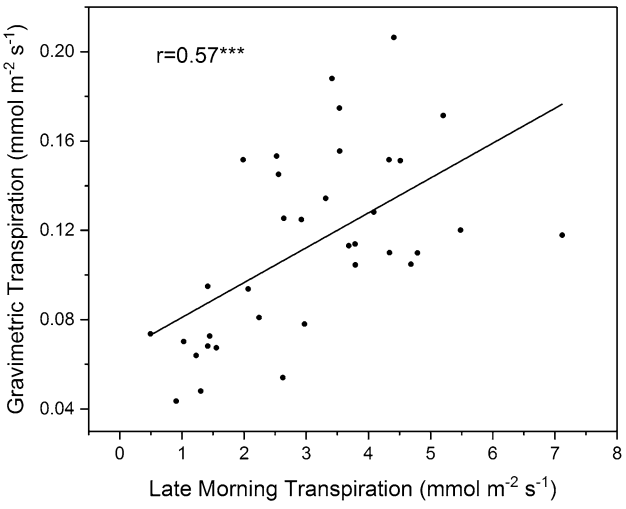

Fig. 5 The relationship between whole plant, gravimetrically determined transpiration and leaf level transpiration rates measured using a Li-Cor 6400XT gas exchange system. ***$P < 0.001$

measured mid-morning was significantly correlated with whole plant daily water use when expressed as a function of leaf area (Fig. 5). Leaf level transpiration was an order of magnitude higher than whole plant water use when using the same units.

Observed decrease in stomatal conductance after the application of exogenous ABA to treated trees also caused a decrease in leaf photosynthetic rates ($P < 0.05$). Photosynthetic rates ranged from 13 to 19 µmol CO_2 m^{-2} s^{-1} for the untreated control and between 10 and 13 µmol CO_2 m^{-2} s^{-1} for ABA-treated trees (Fig. 4). Prior to treatment with ABA, photosynthetic rates were not significantly different between treatments. Immediately after application, late morning photosynthetic rates decreased by approximately 30% in ABA-treated trees compared to untreated trees (Fig. 4).

Whole plant biomass was not significantly different between treated and untreated trees after 90 days of growth (Table 1). However, leaf biomass was significantly greater for the untreated trees compared to trees treated with ABA (Table 1). Leaf and root calcium concentrations were not significantly different. Here, only stem calcium concentrations differed significantly between ABA-treated trees and the untreated control ($P < 0.05$; Table 2). The $\delta^{44}Ca$ of the potting media was −4‰ ($N = 5$). When the tracer was added, the mean potting media $\delta^{44}Ca$ increased to approximately 40‰ (data not shown). For trees that did not have $^{44}CaCO_3$ added to them, the $\delta^{44}Ca$ value was −2.1, 4.1, and 2.4‰ for roots, stems, and leaves, respectively (Fig. 6). The addition of ^{44}CaCO3 to the pots resulted in a significant increase in plant $\delta^{44}Ca$ relative to the trees that did not have $^{44}CaCO_3$ applied to them (Fig. 6) As expected, roots were more enriched than stems and stems were more enriched than leaves. However, root, stem, or leaf ^{44}Ca was not significantly different between the control and ABA treatments. Root, stem, and leaf $\delta^{44}Ca$ averaged 20.4, 11.3, and 5.7‰, respectively (Fig. 6). Almost half of the tracer was accounted for when analyzing both the potting media and the plant (Table 3) indicating that some of the tracer was either flushed into the saucer under the pot or bound in non-exchangeable fractions in the potting media. There was no difference in overall uptake efficiency between treatments where 25.4 and 26.7% of ^{44}Ca was taken up by untreated trees and ABA-treated trees, respectively. When calculating the amount of tracer in each plant organ, the amount of ^{44}Ca in roots was greater for trees that were treated with ABA (Table 3). This was counter-balanced by an associated reduction in ^{44}Ca recovery in the stems of those same trees.

Table 1 Mean root, leaf, stem, and total biomass of ABA-treated and control 'Honeycrisp' apple trees after 90 days of growth

	Leaf (g)	Root (g)	Stem (g)	Total biomass (g)
Control	51.22 ± 2.74a	63.55 ± 5.04a	253.78 ± 6.28a	368.55 ± 18.48a
ABA	31.43 ± 1.71b	52.99 ± 7.68a	255.26 ± 5.49a	339.68 ± 12.26a

Letters denote mean separation between treatments using Tukey's HSD test ($\alpha = 0.05$)

Table 2 Mean calcium concentrations (% w/w ± SEM; $N = 5$) for leaves, stems, and roots of ABA-treated and control 'Honeycrisp' apple trees after 30 days of treatments and 90 days of growth

	Calcium content (% w/w)		
	Leaves	Stems	Roots
Honeycrisp			
Control	0.94 ± 0.06a	0.91 ± 0.06a	2.16 ± 0.12a
ABA	0.92 ± 0.03a	0.81 ± 0.06b	2.22 ± 0.08a

Letters denote mean separation between treatments using Tukey's HSD test ($\alpha = 0.05$)

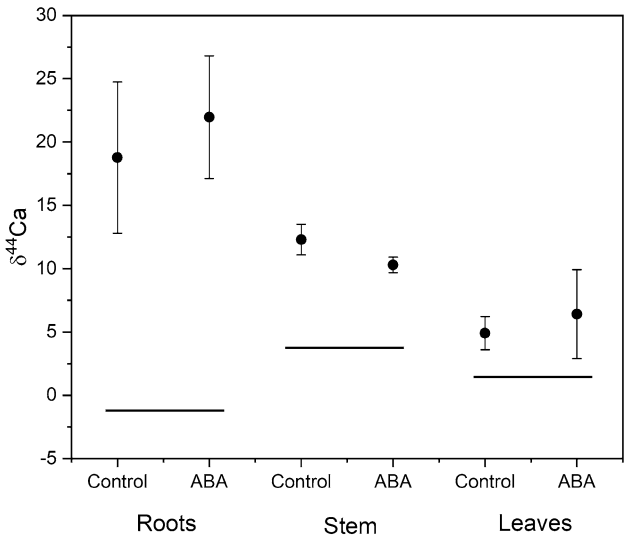

Fig. 6 Mean leaf, stem, and root $\delta^{44}Ca$ of ABA-treated and control 'Honeycrisp' apple trees ($N = 5$) at 30 days after the application of ^{44}Ca stable isotope tracer to the potting media; error bars denote standard error of the means ($N = 5$) and horizontal lines represent the mean $\delta^{44}Ca$ of roots, stems, and leaves from trees without $^{44}CaCO_3$ added ($N = 10$)

The amount of ^{44}Ca recovered in leaves was not different between treatments. During the 30-day experimental period, less than 40% of the ^{44}Ca taken up into the tree stayed in the roots for untreated trees. Whereas more than 50% of the applied ^{44}Ca stayed in the roots for ABA-treated trees (Fig. 7).

Discussion

ABA reduced plant water use at the whole plant and leaf level

The daily variability in gravimetric water use over the course of the experiment was positively related to the light intensity. Since the light conditions were ambient and the temperatures in the greenhouse was controlled relative to external conditions, light was a significant driver of plant water use, where low-light conditions are usually accompanied by low VPD. Therefore, water use would be lower under conditions of low light. Similar results were reported in cherry (Centritto et al. 2000) and kiwifruit (Montanaro et al. 2006). The application of ABA at 500 mg L^{-1} reduced plant water use by approximately 40%. Here, stomatal conductance for ABA-treated trees was approximately 60% lower than the untreated control (Fig. 4) and was consistent with previous studies using synthetic ABA on apple (McArtney et al. 2014). Here, the effect of ABA on potted trees lasted a similar duration as reported by McArtney et al. (2014), where there was ABA-induced stomatal closure within three hours of ABA application at a concentration of 250 mg L^{-1} and the effect lasted for 21 or more days. Here, higher rates of ABA were applied and 30 days after application, transpiration was still lower in the ABA-treated trees than the control.

Changes to transpiration affects calcium partitioning to shoots

Total calcium levels were not lower in plants where transpiration was reduced through the application of ABA. However, there was an accumulation of calcium in the roots of trees treated with ABA. De Freitas et al. (2014) observed a decrease in calcium concentrations in tomato leaves when treated with ABA. However, these tomato plants had fruit and this may have affected the source: sink balance rather than the net accumulation in the aboveground portions but calcium concentrations in the roots were not measured so total plant calcium could not be assessed in this case. The association between transpiration and calcium allocation is mainly supported by the observations of the uneven distribution of calcium between leaves and low-transpiring

Table 3 ^{44}Ca tracer (mg) present in soil, root, stem, leaf, and shoot (stem + leaf) fractions and percent tracer recovery and uptake for 'Honeycrisp' apple trees treated with ABA compared to an untreated control

	^{44}Ca added (mg)	^{44}Ca in soil extraction (mg)	^{44}Ca in roots (mg)	^{44}Ca in stems (mg)	^{44}Ca in leaves (mg)	^{44}Ca in shoots (mg)	% recovery	% uptake
Control	8	1.59 ± 0.34a	0.55 ± 0.10a	1.13 ± 0.25b	0.05 ± 0.03a	1.18 ± 0.21b	41.5 ± 2.6a	21.6 ± 3.7a
ABA	8	1.53 ± 0.24a	0.92 ± 0.17b	0.78 ± 0.08a	0.05 ± 0.03a	0.83 ± 0.21a	41.0 ± 2.7a	21.8 ± 2.0a

% recovery is equal to the entire pool recovered in the analysis including ammonium acetate soil extractions and % uptake indicates the percentage of tracer taken up into the plant over a 30 day period

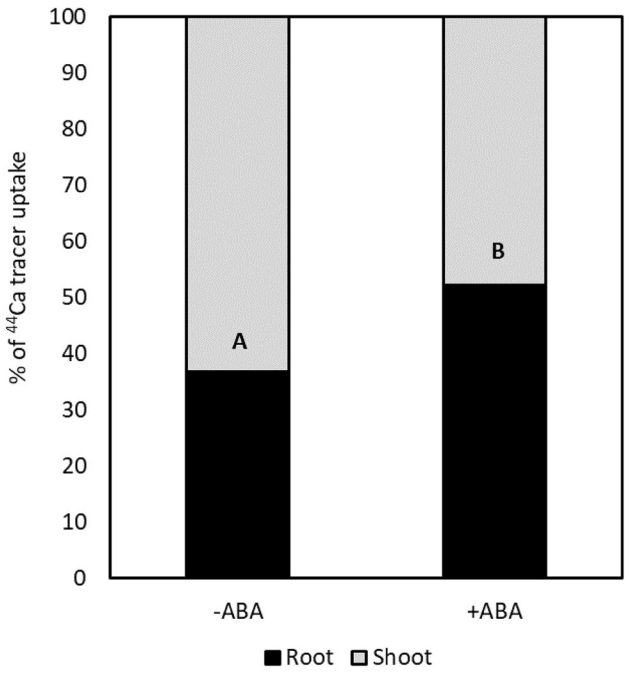

Fig. 7 The allocation of ^{44}Ca between below- and aboveground fractions of 'Honeycrisp' apple trees sampled 30 days after being treated with ABA (ABA+) or left untreated (ABA−). Letters denote significant difference between means determined using a Tukey's HSD test ($\alpha = 0.05$)

fruits where leaf calcium concentrations are much greater than fruit (Dayod et al. 2010; Montanaro et al. 2014; Falchi et al. 2017). In the present study, a direct association between transpiration rates and overall calcium partitioning to the shoot was observed. While endogenous ABA has been attributed to stress responses in plants and changes in root growth, the influence of exogenous ABA on root growth has been less described. Here, there did not appear to be any major influence of foliar-applied ABA on root traits. Further supporting this hypothesis, root uptake did not appear to be impacted since overall tracer uptake over 60 days was unchanged. Calcium continued to accumulate more proportionately in the roots. Although this was not evident when just looking at δ^{44}Ca of roots, stems, and leaves (Fig. 3), the integration of δ^{44}Ca, calcium concentration, and plant biomass showed an increase in overall ^{44}Ca present in the roots at the end of the study. Here, we provide evidence that decrease in whole plant transpiration rates decreased the flow of calcium to the shoots and increased the proportion of calcium remaining in the roots (Fig. 7).

^{44}Ca stable isotope tracer for long- and short-term calcium uptake studies in perennial plants

Here, only small levels of enrichment were detected in leaves after 30 days of tracer application (Fig. 6). When tracing calcium fluxes in the plant soil system with a stable isotope tracer, the isotopic ratio of the enriched material introduced into the system should be diluted over time as a function of (1) the amount of calcium initially present in the system, (2) the input flux of "natural abundance" calcium over the experiment and (3) the output flux of stable isotope tracer over the experiment. The amount of applied isotope tracer is therefore an important experimental parameter and must be sufficiently high to change the isotopic signature of the different plant tissues (enough to analytically be able to resolve differences between control and treatment samples) but sufficiently low to avoid the introduction of calcium fertilization effects.

In our case, the δ^{44}Ca of the potting media increased to 40‰ after the addition of 20 mg of $^{44}CaCO_3$ (97% atom^{44}Ca) tracer. The strong isotopic dilution observed after the soil labeling is due to a substantial amount of calcium initially present in the soil (approximately 4 g of exchangeable calcium was present in the potting media at the time of ^{44}Ca addition). Reducing the calcium pool in the potting media prior to the addition of the labeling tracer could have increased the contrast of the tracer but could have had unintended consequences on plant growth due to calcium deficiencies or imbalances with other nutrients in the potting media. Increasing the isotopic tracer amount and the ^{44}Ca/^{40}Ca ratio in the leaves may provide sufficient contrast to detect the presence of calcium tracers in this compartment but cost limitation would reduce the practicality of this approach. The low recovery of calcium

tracers in the leaves may also be explained by the slow transport of calcium in the xylem stream (Augusto et al. 2011; van der Heijden et al. 2015). In an in situ magnesium and calcium isotopic tracing experiment (tracers were applied to the forest floor) carried out in a 35-year-old European beech tree forest stand, van der Heijden et al. (2015) did not observe any tracers in the canopy compartment during the two first years of monitoring of the experiment. In future soil-based tracing experiments, cost will continue to be a limiting factor in the use of ^{44}Ca as a stable isotope tracer. It will be important to balance cost with the ability to track the presence of the tracer in different plant tissues.

Conclusion

Here, we demonstrate the use of ^{44}Ca as a physiological tracer to better understand the association between transpiration and calcium uptake and allocation in apple trees. Whole plant calcium uptake was not affected when transpiration was reduced using exogenously applied ABA. However, the allocation of calcium from the roots to the shoot decreased with transpiration demonstrating that partitioning of calcium pools between below- and aboveground portions of the plant are affected by transpiration flows and water demand by leaves. We acknowledge the additional questions surrounding the long-term residual effects of higher calcium concentrations in roots on calcium movement to the aboveground portions that were not able to be addressed with these short-term experiments. Specifically, what happens to calcium accumulating in the root during periods of low shoot transpiration and is this calcium pool mobile? Through the further development of the use of ^{44}Ca tracers for use in short- and long-term experiments, there are increasing opportunities to better understand source–sink relationships in calcium movement in horticultural species where calcium-related disorders continue to cause high economic losses.

Author contribution statement LK, GVDH, and LG designed the experiment. LK, GVDH, and LG conducted the experiment. LK, GVDH, and LG analyzed the results. LK, SW, and GVDH wrote and revised the final version of the article.

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