Shoot-derived abscisic acid promotes root growth

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ABSTRACT

The phytohormone abscisic acid (ABA) plays a major role in regulating root growth. Most work to date has investigated the influence of root-sourced ABA on root growth during water stress. Here, we tested whether foliage-derived ABA could be transported to the roots, and whether this foliage-derived ABA had an influence on root growth under well-watered conditions. Using both application studies of deuterium-labelled ABA and reciprocal grafting between wild-type and ABA-biosynthetic mutant plants, we show that both ABA levels in the roots and root growth in representative angiosperms are controlled by ABA synthesized in the leaves rather than sourced from the roots. Foliage-derived ABA was found to promote root growth relative to shoot growth but to inhibit the development of lateral roots. Increased root auxin (IAA) levels in plants with ABA-deficient scions suggest that foliage-derived ABA inhibits root growth through the root growth-inhibitor IAA. These results highlight the physiological and morphological importance, beyond the control of stomata, of foliage-derived ABA. The use of foliar ABA as a signal for root growth has important implications for regulating root to shoot growth under normal conditions and suggests that leaf rather than root hydration is the main signal for regulating plant responses to moisture.

Key-words: abscisic acid (ABA); auxin (IAA); lateral roots; pea; shoot-to-root signalling; tomato.

INTRODUCTION

The phytohormone abscisic acid (ABA) is a key regulator of the physiological responses of plants to water deficit. The best-studied of these responses is the closure of seed-plant stomata during drought stress (Mittelheuser and Van Steveninck, 1969; Geiger et al. 2011), yet one of the most easily measured effects of ABA is the pronounced inhibition of growth in all plant tissues after exogenous application (Milbornow 1974). The inhibitory effect of endogenous ABA levels on shoot growth is obvious and ubiquitous and thought to largely stem from the induction of stomatal closure and resultant decreases in assimilation (Tardieu et al. 2010). However, when shoot growth is measured under strictly controlled conditions that do not result in differences in leaf turgor between ABA-biosynthetic mutants and wild-type plants, ABA-deficient plants show either no inhibition or a promotion of shoot growth, compared with the wild-type plants, which suggests an influence on growth independent of differences in leaf water status (Chen et al. 2003; Ruggiero et al. 2004). In contrast to the inhibitory effect on shoot growth, increased levels of endogenous ABA in plants experiencing stressful conditions promote root elongation (Creelman et al. 1990; Saab et al. 1990; Sharp et al. 1994) in preference to lateral root formation (De Smet et al. 2003; Deak and Malamy 2005). These differential changes in growth by ABA are thought to be an adaptive response to water stress, increasing the allocation of biomass to roots and thereby the effectiveness of root water uptake (Creelman et al. 1990).

The mechanisms responsible for the effect of ABA on root growth are complex, involving interactions and signalling through other phytohormones. Ethylene is central to the influence of ABA on root growth. Early work using ABA-biosynthetic mutants found that ethylene levels in roots were much higher in the absence of ABA (Sharp and Le Noble 2002). Through the use of ethylene and ABA-biosynthetic inhibitors, it was shown that high levels of ethylene in the roots of ABA-deficient plants were the primary cause of reduced root growth (Spollen et al. 2000). These observations have subsequently been confirmed in ABA-biosynthetic mutants across a diversity of species (Cheng et al. 2002). More recently, it has been suggested that ethylene limits root growth by its effect on the hormone auxin, with auxin acting as the primary inhibitor of root growth (Růžička et al. 2007; Swarup et al. 2007). Thus, high ABA levels suppress ethylene synthesis, which in turn reduces auxin transport and biosynthesis in the root tip, removing this primary inhibitor of root growth, and thereby promoting root growth. Increased ABA levels, both directly and indirectly (through both auxin-dependent and auxin-independent mechanisms), reduce lateral root formation (De Smet et al. 2003; Deak and Malamy 2005).

For decades, research into the regulatory role of ABA has focused on responses to soil water deficit, based on the critical assumption that the pool of ABA driving both stomatal aperture and root developmental effects is large and root-derived. There was a prevailing view that root tips are the primary site of ABA biosynthesis when a plant experiences water deficit (Zhang et al. 1987; Zhang and Davies 1989). Indeed, there have been many reports concerning the movement of ABA from the roots to the leaves through the xylem and the effect of this ABA pool on stomatal aperture during drought (Davies and Zhang 1991; Zhang and Davies 1991; Tardieu and Davies
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1993). More recently, however, this idea has been challenged with evidence that leaves synthesize the bulk of the ABA involved in stomatal regulation. During water limitation, unlike roots (Ernst et al. 2010), leaves express the full suite of ABA-biosynthetic genes in the vasculature (Christmann et al. 2005; Okamoto et al. 2009) and have the capacity to transport this ABA to the site of action, the stomata (Kuromori et al. 2014). In addition, small but physiologically significant changes in ABA synthesized in the leaf occur dynamically in well-watered angiosperms, in response to changes in humidity (McAdam and Brodribb 2015; McAdam et al. 2015a). These dynamic fluctuations are responsible for stomatal responses and might have an influence on plant growth. The possibility that fluctuations in foliar-derived ABA can influence whole plant growth is supported by studies reporting substantial export of ABA synthesized in the leaves to other tissues, especially roots, via the phloem (Setter et al. 1980; Setter and Brun 1981; Zhong et al. 1996; Jeschke et al. 1997). In addition, a single study in Arabidopsis thaliana reported that labelled ABA can be transported from the leaves to the roots, via the phloem, although this only occurred when plants experienced water limitation (Ikegami et al. 2009).

Reciprocal grafting studies are a highly effective method for observing the effects of ABA transport around the plant body (Holbrook et al. 2002; Chen et al. 2003; Christmann et al. 2007). Using reciprocal grafts between the flacc a ABA-biosynthetic mutant and wild-type plants of Solanum lycopersicon (tomato), at a late stage of development, Chen et al. (2002) observed that both ABA levels and root growth in plants with ABA-deficient scions were somewhat reduced compared with plants with wild-type scions. This study raises the possibility that foliage-derived ABA may play a major role in root development in well-watered conditions. Following on from this study, to resolve the issue of whether ABA in the roots of plants is synthesized in situ or is imported from the shoot under well-watered conditions, here we conducted reciprocal grafting experiments between wild-type plants and ABA-biosynthetic mutants in both Pisum sativum (pea) and S. lycopersicon from a very early stage of development. In conducting grafts prior to the expansion of the first true leaf, we were able assign all differences in root phenotype, including lateral and adventitious root development, to the genotype of the scion. In addition, we made physicochemical quantifications, based on mass spectrometry, of both ABA and IAA levels, to test the hypothesis that ABA might inhibit root growth through a signalling pathway that involves both ethylene and auxin as the primary growth inhibitor. As a final experiment, we used deuterium-labelled ABA to determine whether ABA can be transported from the shoots to the roots under well-watered conditions.

MATERIALS AND METHODS

Transport of labelled abscisic acid from leaves to roots

To test whether ABA could be transported from leaves to roots, we chose three species from three important angiosperm clades including Vicia faba cv. Crimson Flowering (Fabaceae), Zea mays cv. Golden Bantum (Poaceae) and Helianthus annuus cv. Yellow Empress (Asteraeace). Plants were grown from seed in 10 cm pots, containing vermiculite, in a growth cabinet (PGC-105; Percival Scientific). Conditions in the growth cabinet were maintained at 23°C/16°C day/night temperatures and a 16 h photoperiod, provided by a mixture of incandescent and fluorescent lights ensuring a minimum 300 μmol quanta m⁻² s⁻¹ at the pot surface. All plants were watered daily and received weekly applications of liquid fertilizer (Aquasol; Hortico). At approximately 1700 h on the 15th day of growth, 200 ng of labelled [²H₆]ABA, dissolved in 200 μL of water containing 1% methanol (v/v), was applied in 10–20 μL droplets across the surface of the first true leaf; at the same time a 200 μL droplet of water containing 1% methanol (v/v) was similarly applied to the leaf surface of control plants. Application in small droplets ensured that all remained on the leaf surface with the liquid drying within approximately 10 min of application. At midday the following day (19 h later), the whole root system was harvested. Vermiculite was removed from the roots by washing in water, after which the root system was briskly chopped and covered in cold (−20°C) 80% methanol in water (v/v) with added butylated hydroxytoluene (BHT, 250 mg L⁻¹). This methanol extract was purified and analysed by ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS) (see succeeding sections).

Grafting, growth conditions and morphological assessment

The plant lines used in the following studies included P. sativum cv. Torsdag (Fabaceae) and the mutant isolate wilty, developed over five generations of introgression from the original wilty line described by Marx (1976), which has a mutation in the short-chain deoxygenase/reductase responsible for the conversion of xanthoxin to abscisic aldehyde (McAdam et al. 2015b); and S. lycopersicon cv. Rhinelands Rhun (Solanaceae) and mutant isolate sitens, which carries mutations in the ABA-specific ALDEHYDE OXIDASE gene responsible for converting abscisic aldehyde to ABA (Harrison et al. 2011). Plants were grown in 14 cm pots containing a 1:1 (v/v) mix of vermiculite and dolerite chips (P. sativum) or a 1:1 (v:v) mix of course river sand and dolerite chips (S. lycopersicon) topped with 4 cm of standard potting mixture. All plants were grown under a 16 h photoperiod (supplemented and extended in the morning and evening by sodium vapour lamps, ensuring a minimum 300 μmol quanta m⁻² s⁻¹ at the leaf surface) and 23°C/16°C day/night temperatures. Relative humidity was maintained at 60% by a dehumidifier with integrated humidity sensors (ADH-1000, Airrex Portable dehumidifier, Hephizbah Co. Ltd.). Grafts (one plant per pot) were made epicotyl to epicotyl (P. sativum) or hypocotyl to hypocotyl (S. lycopersicon) using a wedge grafting technique. All graft combinations were made, including those with effective ABA biosynthesis in the shoots (e.g. wild-type self-grafts and wild-type scions grafted to ABA-biosynthesis mutant stocks); and those without effective ABA-biosynthesis in the shoot (e.g. ABA biosynthesis mutant scions grafted onto wild-type stocks and ABA biosynthesis mutant...
self-grafts). The scion was initially held in place by a small rubber band. Seedlings were grafted 7 days after sowing before there was any macroscopic sign of axillary bud formation (*P. sativum*) or 14 days after sowing before the first true leaves had fully expanded (*S. lycopersicon*). Grafted plants were initially enclosed under a sealed, clear polyethylene terephthalate (PET) bottle terrarium for the first 3 days to maintain a high humidity. Lateral buds of *P. sativum* that grew from the cotyledonary node of the stock were excised on emergence. Slow or weak grafts were excluded from the analysis so that all grafting combinations were represented by seven robust individuals, all with the same number of nodes for morphological analysis, undertaken at 30 (*P. sativum*) or 40 (*S. lycopersicon*) days post-germination. An additional four robust grafts were used for hormone quantification. Root and shoot dry weights were quantified after harvesting and drying at 70 °C for 72 h. Roots were cleaned of all growth medium before drying. During harvesting, an assessment of lateral root formation was made by quantifying the number of lateral roots in the first 30 mm of tap root (*P. sativum*) or the number of adventitious roots on the stem (*S. lycopersicon*) on each individual.

**Abscisic acid and auxin quantification**

Tissue for quantifying the level of ABA in roots and leaves and auxin (IAA) in roots of grafted plants was harvested, weighed (±0.0001 g), chopped and covered in cold (−20 °C) 80% methanol in water (v/v) with added BHT. For root ABA and IAA levels, the whole root system was taken for analysis, following washing and carefully removing all growing medium from the roots. Extraction, purification and physicochemical quantification of ABA and IAA by UPLC-MS with an added internal standard were undertaken according to the methods of McAdam and Brodribb (2014) for ABA and Lam *et al.* (2015) for IAA. Samples collected to determine the presence of [6H6]ABA in roots following feeding were also purified as described by McAdam and Brodribb (2014).

**Figure 1.** Mean root (a, b) and foliar (c, d) abscisic acid (ABA) levels in reciprocal grafts of wild-type and isogenic ABA-biosynthetic mutants *wilty* of *Pisum sativum* and *sitiens* of *Solanum lycopersicon* (*n* = 4, ±SE). Genotypes of scions are shown above the genotype of the stock. Different letters denote significant differences between means.

Statistical analysis

Differences in the ABA levels and growth traits measured between the reciprocal graft combinations were tested by one-way analysis of variance (ANOVA). Pair-wise comparisons were carried out using Tukey’s test. Analyses were performed using R Statistical Software.

RESULTS

Transport of abscisic acid from shoots to roots

In three herbaceous angiosperm species chosen from three major angiosperm clades, including a monocot, rosid and asterid, we found that deuterium-labelled ABA could be readily transported from the leaves to the roots under normal, unstressed conditions. After 19 h following the application of a relatively small quantity of [\(^2\)H\(_6\)]ABA to the surface of leaves, this compound could be readily detected in root tissue by UPLC-MS analysis (Supporting Information Fig. S1).

Reciprocal grafting

To determine the origin of ABA involved in regulating root growth and architecture, we made reciprocal grafts between wild-type plants and ABA-biosynthetic mutants, using two classical ABA-biosynthetic mutants, *wilty* of *P. sativum* and *sitiens* of *S. lycopersicon*, to create individuals that were less capable of synthesising ABA in one part of the plant: either the root or the shoot. Reciprocal grafting revealed that ABA in the roots of unstressed plants originated in the shoots and was transported basipetally. Reciprocal grafts between wild-type and ABA-biosynthetic mutant plants indicated a distinct shoot-dominated primary location for ABA biosynthesis. In both *P. sativum* and *S. lycopersicon*, ABA levels in roots were dramatically lower in plants that had an ABA-biosynthetic mutant scion (77 to 93% less, respectively), even if the roots carried the wild-type version of the gene (Fig. 1). In contrast, ABA levels in the shoot were only significantly reduced compared with wild-type controls if the shoot itself was an ABA-biosynthetic mutant (Fig. 1). Wild-type scions grafted onto ABA-biosynthetic mutant stocks showed no significant reduction in ABA levels across the whole plant (Fig. 1). Thus, in grafted plants, ABA levels in both the root and the shoot were determined by ABA synthesis in the shoot.

Root morphological influence of shoot-derived abscisic acid

Shoot-derived ABA had a major influence on the root morphology of plants grown under well-watered, unstressed conditions. In both species, plants with ABA-biosynthetic mutant scions (and therefore lower root ABA levels) had significantly reduced root biomass, regardless of the genotype of the roots (Fig. 2). This biomass reduction was more pronounced in roots than in the shoot, leading to significantly lower root to shoot ratios, in all plants with ABA biosynthesis mutant scions (Fig. 3). In contrast, plants with wild-type scions (and therefore normal root ABA levels) had significantly greater root weights and root to shoot ratios, regardless of whether they were self-grafted or grafted to ABA-biosynthetic mutant stocks (Figs 2 & 3). In addition, shoot-derived ABA had a significant influence over root form. Normal levels of ABA from the shoot suppressed the formation of lateral roots (Fig. 4). Plants that had ABA-biosynthetic mutant scions had double the number of lateral roots in the first 30 mm of the main tap root in *P. sativum* and 15-fold more adventitious roots in *S. lycopersicon* (Fig. 4). The influence of ABA on root morphology was entirely dependent on ABA derived from the shoot and not from ABA synthesized

in root tissue under well-watered, unstressed conditions (Fig. 2). Shoot growth was not affected by an inability of roots to synthesize ABA in either species; plants with wild-type scions having the same number of nodes and dry shoot mass (Fig. 2). The effect of the *wilty* mutation on ABA levels in *P. sativum* was less severe than the effect of the *sitiens* mutation on ABA levels in *S. lycopersicon* (Fig. 1). This smaller difference in ABA levels between the *wilty* mutant and the wild-type in *P. sativum* was reflected in less pronounced phenotypes in the reciprocal grafts of *P. sativum* compared with *S. lycopersicon* (Figs 2, 3 & 4).

**Figure 3.** Mean root to shoot ratio in reciprocal grafts of wild-type and isogenic abscisic acid (ABA)-biosynthetic mutants *wilty* of *Pisum sativum* (a) and *sitiens* of *Solanum lycopersicon* (b) (*n* = 7, ±SE). Genotypes of scions are shown above the genotype of the stock. Different letters denote significant differences between means. Representative images of roots for each reciprocal graft combination and scale bars are shown.

**Figure 4.** Mean number of lateral roots in the first 30 mm of tap root and mean number of adventitious roots in reciprocal grafts of wild-type and isogenic abscisic acid (ABA)-biosynthetic mutants *wilty* of *Pisum sativum* (a) and *sitiens* of *Solanum lycopersicon* (b), respectively (*n* = 7, ±SE). Genotypes of scions are shown above the genotype of the stock. Different letters denote significant differences between means. Representative images of the root or stem for each reciprocal graft combination and scale bars are shown.
Root auxin levels

Differences in the ABA levels measured in the roots of reciprocal grafts corresponded with significant differences in the IAA levels of roots (Supporting Information Fig. S2). Plants that had low ABA levels in the roots, reduced root growth and greater number of lateral or adventitious roots were found to have significantly higher root IAA levels compared with plants that had high ABA levels in the roots and greater root growth (Supporting Information Fig. S2).

DISCUSSION

Shoot-derived abscisic acid influences root growth

Here, we present evidence that most of the ABA found in the root is synthesized in the shoot and then transported to the root system. This foliage-derived ABA has the same effect of promoting root growth and inhibiting lateral root development as demonstrated for root-derived ABA in wild-type plants when soil water is limiting (Sharp and LeNoble 2002). To date, almost all of the work investigating the coordination of shoot and root growth by ABA has focused on the major changes in ABA level that occur in plants in response to soil water deficit (Munns and Cramer 1996; Spollen et al. 2000). This work has largely assumed that the elevated levels of ABA responsible for root morphological change under stress are synthesized by the roots themselves. In contrast to this assumption, a recent study found that foliar ABA levels in angiosperms fluctuate dynamically in response to changes in humidity, and that these fluctuations were responsible for stomatal closure (McAdam and Brodribb 2015). These findings indicate that foliar ABA is synthesized in the leaves and that dynamic fluctuations in foliar ABA level in response to changes in humidity are easily within the magnitude of variation that can have a major influence over root phenotypes, as indicated here by the morphological effects of ABA-deficient mutants. The hypothesized influence on root growth and architecture by ABA synthesized in the leaves in response to humidity changes represents an undescribed means by which plants could regulate root growth under well-watered conditions. ABA, acting as a shoot-derived signal, which is enhanced when leaves experience increasing atmospheric water deficits, may be very important for coordinating water uptake and water use, by regulating root to shoot ratios.

In our study, grafts of both species were made at a stage of development before the shoot could play a significant role in the development of roots, being prior to the expansion of the first true leaf. Using this method, we observed a greater effect of ABA derived from the shoots on the growth and development of roots than that observed by Chen et al. (2002), who undertook reciprocal grafts of another ABA-biosynthetic mutant in S. lycopersicon 30 days after germination; a period of time more than sufficient for substantial root development to have already taken place. This may explain why Chen et al. (2002) did not report on the striking differences in adventitious root development that we observed in our reciprocal grafts of S. lycopersicon.

Our observation of high IAA levels in the roots of plants with ABA-deficient scions suggests that shoot-derived ABA promotes root growth and inhibits the development of lateral or adventitious roots through an ethylene and auxin-mediated pathway. With IAA known to act as a strong inhibitor of root growth and ABA shown to inhibit the production of ethylene in roots, which would in turn reduce the production of IAA (Spollen et al. 2000; Růžička et al. 2007; Swarup et al. 2007), our measurements of endogenous auxin content and root growth appear to support this theory.

Abscisic acid levels in both roots and shoots are strongly influenced by the shoot

In three diverse angiosperm species, we found that labelled ABA can be translocated from the shoots to the roots under well-watered conditions (Supporting Information Fig. S1). This result supports a previous finding of Ikegami et al. (2009) that \(^{13}\)C-labelled ABA was transported from the leaves to the roots in Arabidopsis thaliana in response to water deficit. However, unlike Ikegami et al. (2009), we found that \(^{3}\)HABA applied to leaves was readily transported to the roots in well-watered plants, and this transport did not require a stressed leaf or root water deficit. It has been shown in a number of species that labelled ABA applied to unstressed leaves can be readily transported in the phloem, and as a result is speculated to be transported to sinks including young leaves and roots (Zeevaart and Boyer 1984). Our results clearly support the theory that the shoots are the main source of ABA in the plant (Holbrook et al. 2002; Christmann et al. 2005). Reciprocal grafting, followed by ABA quantification, provides definitive evidence.

The ability of plants to transport ABA from leaf tissue to roots under well-watered conditions was clearly shown here in reciprocal grafts of wild-type and ABA-biosynthetic mutant plants (Fig. 1). Our results are at odds with the widely held view that ABA synthesis in the root contributes almost all of the physiologically relevant ABA in the plant (Zhang et al. 1987). Consistent with our results, a minimal role of root-sourced ABA in mediating whole plant physiology is highlighted by both molecular and drought physiological studies (Holbrook et al. 2002; Christmann et al. 2007; Ernst et al. 2010). ABA synthesis in roots has been shown to occur only after stomata have closed during drought (Ernst et al. 2010); and an inability of roots to synthesize ABA has no effect on the ability of stomata to respond to water stress in plants that have a shoot system that can synthesize ABA during drought (Holbrook et al. 2002; Christmann et al. 2007).

Conclusion

In this study, we show that foliar ABA can be transported to roots under well-watered conditions, and that foliar ABA levels have a major influence over the levels of ABA in roots. In addition, foliar-derived ABA has a profound influence over root growth in well-watered plants. This may be through a mechanism that involves the root growth-inhibiting hormone
IAA. The control of root growth and architecture by foliage-derived ABA may be a very important mechanism by which plants regulate the ratio of root to shoot investment under normal conditions. As a future study, it will be important to investigate whether the more dramatic increases of foliage-derived ABA, which occur during drought stress, play an even greater role in determining root growth and development.

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**SUPPORTING INFORMATION**

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

**Figure S1.** Figure S1. The detection of $[^2H_6]$ABA in the roots of three representative angiosperm species following foliar application.

**Figure S2.** Root IAA levels in reciprocal grafts between ABA-deficient mutant and wild-type plants in *P. sativum* and *S. lycopersicon.*